# STUDIES ON STAPHYLOCOCCI FROM THE BOVINE UDDER

## I. BIOLOGICAL CHARACTERISTICS OF STAPHYLOCOCCI AND SOME OBSERVATIONS ON THE PATHOGENIC STRAINS

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### INTRODUCTION

Many tests as criteria for defining the pathogenicity of staphylococci have been reported. However, there is still lack of information pertaining to the most suitable methods for their recognition.

In 1955, a bacteriological survery on bovine mastitis in Hokkaido was conducted by HIRATO et al. Milk samples from 801 cows from 4 different areas (Yakumo, Hayakita, Obihiro and Okoppe) were examined. From this survey, many cases of the staphylococcal mastitis were observed as has already been reported.

The present author became particularly interested in establishing criteria for the differentiation of these pathogenic strains from non-pathogenic varieties. Accordingly the micrococci from the bovine udder as described above have been subjected to a number of cultural tests as well as to phage typing.

The present report will describe the production of pigment, coagulase and hemolysin, enterotoxin and the phage types of the isolated strains.

### MATERIALS AND METHODS

*Strains examined* Six hundred and seventy-eight strains of micrococci were isolated from the milk samples of 473 cows (674 quarters) among 801 which were examined for mastitis in four different areas in Hokkaido during the period from April to June, 1955.

One loopful (0.05 ml) each of fresh milk sample was cultivated on a sheep or horse blood agar plate. After 24~48 hours incubation at 37°C, developing colonies were examined for micrococci; a total 678 strains were isolated.

The biological characteristics of these 678 strains were carefully examined following "BERGEY's Manual of Determinative Bacteriology" (1948), namely, pigmentation, gelatin liquefaction, nitrates reduction, utilization of $NH_4H_2PO_4$, the fermentation of lactose and mannitol and reactions in litmus milk and so on (Table 1). From these tests, the most of

TABLE 1. *Result of Identification of Micrococci from Milk* (following BERGEY's Manual, 6th Edition)

| TYPE OF ORGANISMS | BIOLOGICAL CHARACTERS OF TYPICAL STRAINS | | | | | | | NO. OF STRAINS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pigment | Liquefaction of gelatin | Nitrates reduction | $NH_4H_2PO_4$ | Fermentation of Mannitol | Fermentation of Lactose | Litmus milk | Typical | Atypical | Total |
| *M. pyogenes* var. *aureus* | orange | + | + | − | + or − | + | A.C.P.* | 229 | 107 | 336 |
| *M. pyogenes* var. *albus* | white | + | + | − | + or − | + | A.C.P. or A.C. | 139 | 24 | 163 |
| *M. citreus* | lemon yellow | + | + | − | + or − | + | A.C. | 12 | 4 | 16 |
| *M. aurantiacus* | orange to white | + ≀ − | + | − | − | + | A. | 54 | 25 | 79 |
| *M. epidermidis* | white | − ≀ + | + | − | − | + | A. | 9 | 6 | 15 |
| *M. candidus* | — | − | − | − | | + | A. | 7 | 4 | 11 |
| *M. flavus* | yellow | + | − | − | − or + | + | A.C.P. | 8 | 3 | 11 |
| *M. conglomeratus* | yellow | + | + | + | − | + | A. | 3 | 7 | 10 |
| *M. varians* | yellow | − | + | + | + or − | + | A. | 6 | 1 | 7 |
| *M. caseolyticus* | — | + | + or − | + | + | + | A.P. | 9 | 16 | 25 |
| *M. luteus* | yellow | − | − | + | + | − | A. | 2 | 1 | 3 |
| *M. roseus* | rose red | + | + | + | + | | − or Alk. | 0 | 2 | 2 |

* A.: Acid production C.: Acid curd P.: Proteolysis

the strains were classified as *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*), var. *albus* (*Staphylococcus albus*) and *Micrococcus citreus* (*Staphylococcus citreus*); the remainder were identified as belonging to nine species. The most of the atypical strains of staphylococci in table 1 were those which did not coagulate or peptonize litmus milk, while a few did not ferment lactose.

These 515 strains of staphylococci named above were studied with particular reference to the following properties that had been generally recognized to be the main characters of pathogenic strain. When the strain had been preserved on artificial media for 3 months or more after isolation, they were transferred daily on blood agar plates for 7 days before testing.

*Pigment production* For pigment production, the strains to be tested were streaked on agar plate containing 10% skimmed milk and the pigmentation was recorded after a 4-day incubation period at 22°C. A final recording was further made after the plates had been left at room temperature for 3 days.

*Mannitol fermentation* Each strain was inoculated into a modified BARSIEKOW's medium and observed for 15 days at 37°C.

*Coagulase test* 1) Several kinds of citrated blood plasmas—rabbit, cow, sheep and horse—were employed. These plasmas were diluted by 5 times in physiological saline and 0.5 ml amount of each was placed in small test tubes. Two drops of 24-hour peptone water cultures at 37°C were added to each tube. After shaking thoroughly, the mixtures were allowed to stand in a water-bath at 37°C; results were observed after 30 minutes, 1, 2, 3 and 4 hours after incubation and the final reading was generally made after the tubes had been left overnight at room temperature. The intensity of the reaction was scored as ±±, ±, ±, ± and − according to the firmness of clotting.

2) It was investigated whether or not the rabbit plasma which was mixed with cow, horse or sheep plasma at the rate of 1 : 1, 1 : 4, 1 : 9, 1 : 14 and 1 : 19 respectively and diluted by 5 times in saline could be used for coagulase test instead of rabbit plasma alone. The details for execution are the same as that described in 1).

*Hemolysin production* Five species of blood were used—sheep, cow, horse, rabbit and human. Citrated blood samples of these species were washed three times with saline to remove any anti-hemolysin that might be present in the serum and re-suspended in saline to the original volume of the citrated blood. By using the red cell suspension, 5% blood agar plates (pH 7.6) were prepared. Strains to be examined were streaked on the blood agar plates. Observations were made twice after 24 hours' incubation at 37°C and overnight incubation at 4°C.

*Enterotoxin production* The ability for producing enterotoxin was examined by the method following DOLMAN and WILSON. Portions of DOLMAN's medium were poured into 20 cm petri dishes and 1 to 2 ml of a 5-hour-broth-culture of the test strains were evenly seeded on its surface. The plates were incubated at 37°C for 72 hours in atmosphere containing 20% carbon dioxide. The whole contents of the plates were then squeezed through cheesecloth and centrifuged for 30 minutes at 3,000 r.p.m. The supernatant was placed in a boiling water bath for 20 minutes following Seitz filtration. After removing the resulting precipitate by centrifugation, the clear supernatant was submitted to the

test. Use was made of kittens weighing 200~1,000 g in carrying out the test. The filtrates which had been previously warmed to 37°C were administered intraperitoneally in the proportion of 1 ml per 100 g weight. Animals were closely observed for 4 to 5 hours after injection, and vomiting or diarrhoea was accepted as a positive criterion. Tests were repeated 2 to 3 times with different animals when negative result was obtained at first.

*Bacteriophage typing* The following 20 phages from N.C.T.C. (The National Collection of Type Culture) which were subcultured in National Institute of Animal Health in Tokyo were used for bacteriophage typing. Their grouping were as follows:

Group I : 29, 52 A, 52 and 79.
Group II : 3C, 3B, 3A, 55 and 71.
Group III: 70, 42 E, 6, 7, 73, 47, 54, 75, 18 and 19.
Group IV: 42D.

The technique of phage typings followed was that of FUKUMI, a modification of the method reported by WILLIAMS and RIPPON.

The nutrient agar plates were etched on the back with a grid of 20 squares and flooded with young cultures (4~6 hrs.) of the test organisms. They were left to dry with the lids tilted up for about 30 min. and the phages were then applied with a fine pipette (approx. 100 drops/ml). The dilution of phage filtrate used for routine typing (R.T.D.) is the highest that cause confluent lysis against the propagating strain. When the drops had dried the plates were incubated at 37°C overnight. The scheme of notation was as follows: Confluent lysis with no secondary growth, ±±; confluent lysis with secondary growth, ±; more than 50 plaques, +; 20~50 plaques, +-; less than 20 plaques, ±. The strains which were lysed strongly (i.e. + lysis) by phages were recognized to be susceptible.

## RESULTS AND DISCUSSION

### 1. Pigment Production

Of 515 strains of staphylococci isolated from milk samples, 336 strains (65.3%) produced yellow to orange pigment (*Staph. aureus*), 163 strains (31.7%) white (*Staph. albus*) and the remaining 16 strains (3.1%) lemon yellow (*Staph. citreus*).

Correlations of the pigment production with the coagulase test or hemolysin type are listed in table 2.

CHAPMAN et al.[3] reported that only 51.7% of 690 aureus strains and 13.4% of 1852 albus strains were hemolytic on rabbit blood agar plates. In the present investigation, similar results were also obtained. Forty-four per cent out of 336 aureus strains produced hemolysin, while only 13.5% of albus strains showed positive results in hemolysin test; none of the citreus strains produced hemolysin. Moreover, in strains producing a complex ($\alpha\beta$, $\alpha\delta$, $\beta\delta$ or $\alpha\beta\delta$) hemolysin pattern, the proportion of aureus strains to other pigment producers seemed to be generally higher than those in the strains which have a simple hemolysin pattern ($\alpha$, $\beta$ or $\delta$ only). It was also noteworthy that all the $\alpha\beta\delta$ strains produced orange pigment. In coagulase test, as in hemolysin production, about one half of aureus strains also showed a positive reaction, while most of the albus strains were negative and none of the citreus strains produced coagulase.

TABLE 2. *Relationship between Pigment Production and Hemolysin Type or Coagulase Test*

| PIGMENT | NO. OF STRAINS | HEMOLYSIN TYPE | | | | | | | | COAGULASE | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | α | β | δ | αβ | αδ | βδ | αβδ | Non or Weak | + | − |
| Orange | 336 | 10 | 5 | 8 | 50 | 15 | 35 | 25 | 188 | 166 | 170 |
| White | 163 | 3 | 2 | 1 | 10 | 1 | 5 | 0 | 141 | 23 | 140 |
| Lemon | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 0 | 16 |
| Total | 515 | 13 | 7 | 9 | 60 | 16 | 40 | 25 | 345 | 189 | 326 |

From the data recorded above, there seems to be a considerably close correlation between the pigment formation and coagulase or hemolysin test, but it is not entirely conclusive.

## 2. Fermentation of Mannitol

Three hundred and forty-five strains out of 515 staphylococci fermented mannitol, namely, 75.7% of 336 aureus strains, 47.2% of 163 albus strains and 43.7% of 16 citreus strains.

Nextly, comparison was made between the mannitol fermentation and coagulase production; it was found that almost all coagulase-positive strains (with 2 exceptions) and 158 of 326 coagulase-negative strains fermented mannitol.

Consequently, although nearly all coagulase producers fermented mannitol, the reverse did not hold true of mannitol fermenters.

## 3. Coagulase Test

1) It has been accepted that the coagulase reaction is one of the most reliable tests for the identification of pathogenic staphylococci. However, from the results of different workers it is clear that the reaction is dependent on the kind of plasma used in the test. CRUICKSHANK, CHAPMAN et al.[4], TAYLOR and MCDIARMID[23] and many others maintained that human and rabbit plasmas are most easily clotted by staphylococci.

Therefore, in this study, several kinds of blood plasma viz., rabbit, horse, cow or sheep were compared by using previously selected 215 strains; of them 106 coagulated rabbit plasma and the rest did not. As is clearly noticed in table 3, the best results were obtained in case of the rabbit plasma comparing with the other kinds in respect to number of positive reactions, in time required for clotting and in firmness of the clots. None of cultures which did not coagulate rabbit plasma showed positive results in the other kinds of plasma.

2) With the object for saving rabbit plasma, rabbit plasma samples which were

TABLE 3. *Coagulase Test Using 4 Kinds of Blood Plasma*

| SPECIES OF PLASMAS | COAGULATION OF PLASMA | | | | | TOTAL OF STRAINS |
|---|---|---|---|---|---|---|
| | +++ | ++ | + | ± | − | |
| Rabbit | 86 | 16 | 4 | 0 | 109 | 215 |
| Horse | 17 | 12 | 20 | 2 | 164 | 215 |
| Sheep | 23 | 1 | 8 | 0 | 183 | 215 |
| Cow | 14 | 0 | 1 | 0 | 200 | 215 |

TABLE 4. *Coagulase Test in Mixed Plasmas*

| SPECIES OF PLASMAS | MIXING RATE | NO. OF COAGULASE-POSITIVE STRAINS | | | | TOTAL OF STRAINS |
|---|---|---|---|---|---|---|
| | | +++ | ++ | + | ± | |
| Rabbit and Horse | 1 : 1 | 20 | 1 | 0 | 0 | 21 |
| | 1 : 4 | 23 | 0 | 0 | 0 | 23 |
| | 1 : 9 | 16 | 4 | 1 | 0 | 21 |
| | 1 : 14 | 16 | 6 | 1 | 0 | 23 |
| | 1 : 19 | 18 | 0 | 1 | 0 | 19 |
| Rabbit and Sheep | 1 : 1 | 20 | 1 | 1 | 0 | 22 |
| | 1 : 4 | 21 | 1 | 1 | 0 | 23 |
| | 1 : 9 | 16 | 6 | 0 | 1 | 23 |
| | 1 : 14 | 16 | 6 | 0 | 1 | 23 |
| | 1 : 19 | 20 | 1 | 1 | 1 | 23 |
| Rabbit and Cow | 1 : 1 | 20 | 0 | 0 | 1 | 21 |
| | 1 : 4 | 17 | 0 | 0 | 0 | 17 |
| | 1 : 9 | 12 | 1 | 1 | 0 | 14 |
| | 1 : 14 | 15 | 2 | 0 | 0 | 17 |
| | 1 : 19 | 13 | 3 | 1 | 0 | 17 |
| Rabbit and Saline | 1 : 7 | 21 | 1 | 1 | 0 | 23 |
| | 1 : 19 | 7 | 7 | 9 | 0 | 23 |
| | 1 : 39 | 1 | 6 | 13 | 2 | 22 |
| | 1 : 59 | 0 | 5 | 10 | 0 | 15 |
| | 1 : 79 | 0 | 0 | 6 | 4 | 10 |
| 1 : 4 rabbit plasma | | 21 | 1 | 1 | 0 | 23 |

Note: These plasmas were diluted by 5 times in saline for the test.

prepared by mixing with horse, sheep or cow plasma at the rate of 1 : 1, 1 : 4, 1 : 9, 1 : 14 and 1 : 19 respectively and diluted by 5 times in saline were employed for coagulase test. For this study, 48 strains of which 23 coagulated rabbit plasma and the remainder did not, were chosen. Only the data obtained from the test of 23 strains which coagulated rabbit plasma are listed in table 4.

Rabbit plasma, mixed with that of horse or sheep in the proportion of 1 : 4, gave results comparable with that of rabbit alone regardless of the speed of clotting, but the other mixture were clotted less firmly comparing with rabbit plasma alone. The detail of results will be seen in table 4. Accordingly, it was concluded that the 2 mixed plasmas just mentioned could be substituted for rabbit plasma.

## 4. Hemolysin Production

MINETT[6,17] stated that the great majority of pathogenic udder staphylococci formed $\alpha$ and $\beta$ toxin, and the most of animal staphylococci produced $\beta$ toxin. Furthermore, ELEK and LEVY claimed that $\alpha$- and $\delta$-lysin occurred very frequently in human staphylococci, and that $\beta$-lysin was characteristic of animal strains but uncommon in human strains. On the other hand, WILLIAMS and HARPER reported that $\delta$-lysin was never found alone, but was always combined with $\alpha$- or $\beta$-lysin.

In this report, for purposes of classification of hemolysin type, the terminology used by BRYCE and ROUNTREE[2], MARKS and VAUGHAN[15] and ELEK and LEVY[10] is employed. The characteristics of each hemolysin on blood agar plates were found to be as follows:

$\alpha$ type—A colony which is surrounded by a zone of complete lysis with a hazy indefinite margin on sheep and rabbit blood agar plates after incubation for 24 hours at 37°C (Figs. 1~3).

$\beta$ type—A wide zone of partial hemolysis with a sharply definite margin and is replaced by complete hemolysis on cooling on sheep blood agar plate (Figs. 1~3).

$\delta$ type—A moderately large zone of complete lysis with a sharply definite margin on horse and human blood agar plates (Figs. 1~3).

Furthermore, four complex hemolysin patterns ($\alpha\beta$, $\alpha\delta$, $\beta\delta$ and $\alpha\beta\delta$) were noted according to the combinations of these simple hemolysin patterns.

The comparative value of sheep, rabbit, horse, human and cow blood agar plates for the detection of hemolysin type was determined and the conclusions are listed in table 5.

Either sheep or cow blood appears to be suitable for the detection of $\alpha$ and $\beta$ hemolysins, because the modes of hemolysis on these blood agar plates are peculiar depending on each hemolysin and can be distinguished easily. On the other hand, horse and human erythrocytes are susceptible to $\delta$-lysin, however they are not so affected by the other hemolysins. Accordingly, in this report, the author generally employed sheep, horse and human blood for determining the hemolysin types.

From the tests it was found that 170 out of 515 strains were hemolytic and 13 of them showed $\alpha$-, 7 $\beta$-, 9 $\delta$-, 60 $\alpha\beta$-, 16 $\alpha\delta$-, 40 $\beta\delta$- and 25 $\alpha\beta\delta$-type respectively.

The great majority of these hemolytic strains formed complex hemolysin patterns ($\alpha\beta$, $\alpha\delta$, $\beta\delta$ and $\alpha\beta\delta$); $\beta$-lysin was the most common constituent in these hemolytic strains.

TABLE 5. *Effect of Staphylococcal Hemolysins on Various Species of Blood Agar*

| SPECIES OF BLOOD AGAR | EFFECT PRODUCED BY HEMOLYSIN | | |
|---|---|---|---|
| | α-Lysin | β-Lysin | δ-Lysin |
| Rabbit | Large zone of lysis with hazy margin | Nil | Moderately large zone of lysis with sharp or hazy margin |
| Sheep | As for rabbit red cell | Large sharply defined zone of partial lysis with discoloured cells | Small zone of lysis with sharp margin |
| Horse | Usually nil: occasionally a zone of partial lysis | Nil | Moderately large zone of lysis with sharp margin |
| Human | Small zone of partial lysis, or nil | Small zone of partial lysis, or nil | As for horse blood |
| Cow | Large zone of complete lysis with sharp or hazy margin | As for sheep red cell | Usually as for sheep blood: occasionally nil |

## 5. The Relationship between Hemolytic Activity and Coagulase Production

ELEK and LEVY[10] and SUZUKI[22] reported that coagulase-positive staphylococci were not always hemolytic, but coagulase-negative strains produced none of the hemolysins. However, COWAN[5] and SLANETZ and BARTLEY[20] stated that coagulase production corresponded absolutely with hemolysin production. On the other hand, WILLIAMS and HARPER demonstrated δ-lysin in all coagulase-positive strains which produce either α- or β-lysin or both, while it was not found in coagulase-negative strains.

In the present investigation, the results of examination of all 515 strains showed that all of the hemolytic strains were coagulase-positive except 3 strains of δ type and 2 strains of $\alpha\beta$ type. However, only 24 (7.0%) of 345 weak or non-hemolytic strains were coagulase-positive and the remainder failed to cause coagulation of rabbit plasma (Table 6).

From the data obtained, the following conclusion appears to be justified. Almost all of the staphylococci from milk samples are coagulase-positive if they

TABLE 6. *Relationship between Hemolysin Type and Coagulase Production*

| COAGULASE PRODUCTION | HEMOLYSIS | | | | | | | | TOTAL OF STRAINS |
|---|---|---|---|---|---|---|---|---|---|
| | α | β | δ | αβ | αδ | βδ | αβδ | Non or Weak | |
| + | 13 | 7 | 6 | 58 | 16 | 40 | 25 | 24 | 189 |
| − | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 321 | 326 |

produce α, β or δ hemolysin on blood agar plates, however, a few of the weak or non-hemolytic strains also caused coagulation of rabbit plasma.

## 6. Enterotoxin Production

Twenty-five strains of hemolytic coagulase-positive and 4 strains of weak or non-hemolytic coagulase-negative staphylococci were checked for enterotoxin production.

By referring to table 7 it will be found that all the hemolytic coagulase-positive strains produced enterotoxin except 2 of which one produced β hemolysin and another α and δ hemolysins. On the other hand, there was no evidence of enterotoxin production by any of the non or weak hemolytic coagulase-negative strain tested.

TABLE 7. *Enterotoxin Production*

| COAGULASE | HEMOLYSIS | ENTEROTOXIN PRODUCTION | | TOTAL |
|---|---|---|---|---|
| | | + | − | |
| Positive | α | 2 | 0 | 2 |
| | β | 1 | 1 | 2 |
| | δ | 1 | 0 | 1 |
| | αβ | 10 | 0 | 10 |
| | αδ | 4 | 1 | 5 |
| | βδ | 4 | 0 | 4 |
| | αβδ | 1 | 0 | 1 |
| Negative | Non-hemolysis | 0 | 4 | 4 |

The present result indicates that coagulase and hemolysin appear to be closely associated with the enterotoxigenicity of staphylococci, however, hemolytic or coagulase-positive strains were not always enterotoxigenic.

Recently, HATTA et al. reported that all positive staphylococci in M (mannitol fermentation), C (coagulase production), N (growth in presence of 7.5% NaCl) and H (hemolysis) were enterotoxigenic and others were not. Therefore, for the present report, the correlation between M.C.N.H. and enterotoxigenicity of staphylococci was investigated.

TABLE 8. *Relation between Enterotoxin Production and M.C.N.H.*

| M. C. N. H. | NO. OF STRAINS | ENTEROTOXIN | |
|---|---|---|---|
| | | + | − |
| M. C. N. H.-all positive | 25 | 23 | 2 |
| M. C. N. H.-all positive* | 1 | 1 | 0 |
| M. N.-positive but C.H.-negative | 4 | 0 | 4 |

* This strain was isolated from a rice cake, and showed weak hemolysis under aerobic condition but α hemolysis in atmosphere containing 20% $CO_2$.

As is noticed in table 8, regarding M.C.N.H. all positive strains were not always enterotoxigenic; this fact was somewhat different from that reported by HATTA et al. Furthermore, it is noteworthy that there is one enterotoxigenic strain which did not produce hemolysin in aerobic condition.

## 7. Considerations on the Bacteriological Features Which Were Isolated from the Clinical Mastitis or from the Abnormal Milk Samples

Though much attention has been given to the value of biological tests as criteria for defining the staphylococci which are pathogenic to the bovine udder, no satisfactory method for classification has emerged from the use of these tests. Therefore, in this paper, comparison was made between the following four bacteriological findings which are accepted generally as important criteria of pathogenicity and the abnormality of the udder from which staphylococci had been purely isolated. In this case, udders which showed indurations or positive reactions in strip cup test were regarded as active cases of clinical mastitis.

1) The relationship between pigment production or the fermentation of mannitol and pathogenicity of staphylococci: Out of 37 quarters affected with clinical mastitis, mannitol fermented aureus strains were isolated from 24 quarters, mannitol fermented albus strains from 8, mannitol-negative albus strains from 4 and the remaining one quarter shed mannitol-negative aureus strain. None of citreus strains was demonstrated from any case of clinical mastitis.

Furthermore, these biological properties were compared with cell count of milk samples, except clinical cases. From the data listed in table 9, it will be seen that in the milk samples from the quarter which shed the mannitol-fermenting aureus strains the increase of cell count, more than 500,000, was observed in a rather high rate (55.4%). However, judging from the cell increasing rates, it is very questionable whether very

significant differences may be present or not, as suggested in table 9.

Accordingly, the pigment production or mannitol fermentation cannot be employed as an indication of the pathogenicity.

2) The relationship of hemolysis or coagulase production to the pathogenicity of staphylococci for bovine udder: From their long-term observations on 305 cows, SLANETZ and BARTLEY maintained that staphylococci which produced either acute or chronic mastitis were always hemolytic and coagulase-positive; SCHALM and WOODS, PLASTRIDGE et al. and MINETT had the same opinions. However, DEUBLER and COLE recently stated that because the organisms from the majority of the clinical mastitis cases showed neither hemolysis nor coagulase activity, the relation of these properties to pathogenicity is questionable. On the other hand, HOWARD reported that strains producing a complex hemolysin pattern seem to display greater virulence to mice than strains with a simple hemolysin pattern. Moreover, MARKS and VAUGHAN demonstrated that coagulase-positive staphylococci derived from lesions have a tendency to produce significantly more δ-lysin and α-toxin than those isolated from carriers.

TABLE 9. *Relation of Pigment and Mannitol Fermentation to Cell Count* (with the exception of clinical mastitis)

| PIGMENT | MANNITOL FERMENTATION | NO. OF QUARTERS | PERCENTAGE OF SAMPLES GIVING CELL COUNTS | | | | MEAN CELLS per ml (in thousand) |
|---|---|---|---|---|---|---|---|
| | | | Less than 100,000 | 100,000 ~ 500,000 | 500,000~ 1,000,000 | 1,000,000 or more | |
| Yellow | + | 197 | 16.7 | 27.9 | 24.4 | 31.0 | 1,572 |
| | − | 58 | 34.5 | 31.0 | 15.5 | 19.0 | 861 |
| White | + | 56 | 21.4 | 33.9 | 17.9 | 26.8 | 1,130 |
| | − | 67 | 19.4 | 41.8 | 22.4 | 16.4 | 687 |
| Lemon yellow | + | 6 | 33.3 | 50.0 | 0 | 16.7 | 475 |
| | − | 9 | 33.3 | 33.3 | 33.3 | 0 | 310 |

In order to determine whether hemolysis and coagulase activity are closely related to pathogenicity or not, the author also studied the relationship between these properties and the abnormality of bovine udder. The following conclusions were reached.

Of 37 quarters with clinical mastitis, hemolytic coagulase-positive cultures were isolated from 20 of them (8 out of these yielded strains of $a\beta$-type, 4 $a\beta\delta$-type, 3 $a\delta$-type, 2 $\beta\delta$-type, 2 $\delta$-type and 1 $a$-type) and non-hemolytic coagulase-negative cultures from 17. These data indicate that there are no close correlations between them.

On the other hand, the hemolysin production and the coagulase reaction of the isolated strains were correlated with the cell count of milk samples excepting the clinical cases.

As is indicated in table 10, the cell count of the milk from which hemolytic coagulase-positive cultures were isolated increased comparing with that from which non-hemolytic coagulase-negative strains were isolated. But it is noteworthy that in milk samples from quarters which contained weakly or non-hemolytic coagulase-negative strains, 31.9% or 35.0% of the samples gave cell count of 500,000 or more.

Accordingly, on the basis of these results, although the majority of the hemolytic and coagulase positive strain are pathogenic to bovine udder, it should not be concluded that those which reacted negatively in these properties are non-pathogenic. Further precise experiments should be made to elucidate these points.

Moreover, there seems to be a not very close relationship between the hemolysin type and the cell count of the milk samples.

TABLE 10. *Relation of Hemolysis and Coagulase Production to Cell Count* (with the exception of clinical cases)

| HEMOLYSIS | COAGULASE | NO. OF QUARTERS | PERCENTAGE OF SAMPLES GIVING CELL COUNTS | | | | MEAN CELLS per ml (in thousand |
|---|---|---|---|---|---|---|---|
| | | | Less than 100,000 | 100,000 ~ 500,000 | 500,000~ 1,000,000 | 1,000,000 or more | |
| Non-hemolytic | − | 140 | 25.0 | 40.0 | 19.3 | 15.7 | 723 |
| | + | 5 | 0 | 20.0 | 60.0 | 20.0 | 738 |
| Weakly hemolytic | − | 110 | 30.0 | 38.2 | 16.4 | 15.5 | 685 |
| | + | 15 | 26.7 | 26.7 | 20.0 | 26.7 | 1,092 |
| Hemolytic ($\alpha$, $\beta$, $\delta$) | − | 5 | 0 | 20.0 | 60.0 | 20.0 | 1,108 |
| | + | 127 | 7.0 | 19.7 | 27.0 | 46.4 | 1,935 |

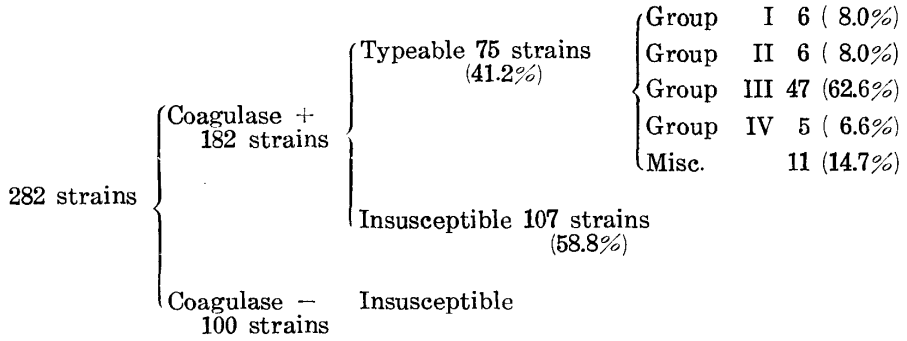
## 8. The Bacteriophage Typing of Staphylococci Isolated from Bovine Milk Samples

Up to date many investigations have been carried out to determine whether the bacteriophage typing could be applied to the classification of staphylococci which originated from animals. SMITH reported that of 1,016 strains isolated from bovine milk samples, 93.3% were phage-typed whilst 6.7% were untypeable. Furthermore, EDWARDS and RIPPON divided the coagulase-positive udder staphylococci into 5 groups by phage typing. However, no satisfactory result generally has been reported from the use of this method.

The author also attempted the phage typing of staphylococci isolated from milk samples by using 182 coagulase-positive strains and 100 coagulase-negative ones.

As shown in table 11, out of 182 coagulase-positive strains, only 75 (41.2%) were phage-typed while 107 (58.8%) were untypeable. Furthermore, 47 (62.6%)

TABLE 11. *Result of Staphylococcal Phage-Typing*




of these 75 typeable strains were susceptible to the phages of Group III and the remainder could be classified into 4 different phage groups. On the other hand, none of 100 coagulase-negative strains proved to be susceptible to any of the test phages.

Thus, although the satisfactory result could not be obtained in this experiment, additional studies are required in which the other phage groups should be used.

## SUMMARY AND CONCLUSIONS

In the present paper, the biological characters, especially criteria for the pathogenicity, of staphylococci of 515 strains encountered in the udder were investigated in detail. The results are summarized as follows:

1. Out of 678 strains of micrococci isolated from milk, 515 were classified as staphylococci and the rest were identified as belonging to nine species, according to BERGEY'S Manual (1948).

2. Out of the 515 strains above mentioned, about 65% were orange pigment producers (*Staph. aureus*), 31% white pigment producers (*Staph. albus*) and 3% lemon yellow pigment producers (*Staph. citreus*).

3. In 336 strains which were classed as *Staph. aureus*, mannitol fermenters were observed in high percentage (75.7%), furthermore, 47.2% of 163 albus strains and 43.7% of 16 citreus strains also fermented mannitol.

4. Coagulase test was carried out on rabbit, horse, sheep and cow plasmas, and it was found that amongst them rabbit plasma was most easily clotted by staphylococci.

5. Rabbit plasma, mixed with that of horse or sheep at the rate of 1 : 4, could be used instead of rabbit plasma alone, but there is tardiness of coagulation.

6. Sheep and cow red cells were most suitable for the detection of $\alpha$- and $\beta$-hemolysin, while for the detection of $\delta$-lysin human or horse blood should be used.

One hundred and seventy strains (33%) out of 515 examined produced hemolysin and the great majority of these hemolytic strains showed complex ($a\beta$, $a\delta$, $\beta\delta$ and $a\beta\delta$) hemolysin patterns. Moreover, $\beta$-hemolysin was demonstrated in most of the hemolytic strains.

7. Almost all hemolytic staphylococci (165 strains out of 170) and 24 (7.0%) of 345 strains showing weak or no hemolysis were coagulase producers.

8. No significant correlation can be suggested between pigment formation or mannitol fermentation and the ability of producing coagulase or hemolysin. Furthermore, pigment production and mannitol fermentation do not serve to distinguish pathogenic strains from non-pathogenic varieties.

9. Enterotoxigenicity was demonstrated in 23 strains of 25 hemolytic and coagulase producers, however, non-hemolytic coagulase-negative 4 strains were not at all enterotoxigenic.

10. The majority of staphylococci giving irritation to bovine udder were hemolytic or coagulase positive, but it cannot be concluded that all strains reacting negatively in these properties are non pathogenic.

11. Seventy-five (41.2%) of 182 coagulase-positive strains were phage-typed, the majority (62.6%)of them were susceptible to the phages of Group III. However, all of 100 coagulase-negative strains were insusceptible to any of the test phages.

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Staphylococcal Hemolysins

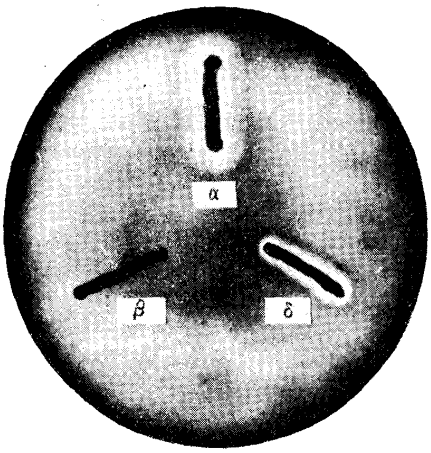


Fig. 1. The mode on rabbit blood agar. α-lysin shows a large zone of lysis with hazy margin, β-lysin nil and δ-lysin moderately large zone of lysis with hazy margin.

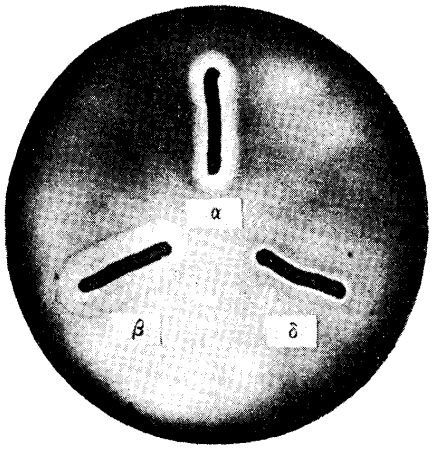


Fig. 2. The mode on sheep blood agar. α-lysin shows a large zone of lysis with hazy margin, β-lysin large sharply-defined zone of partial lysis with discoloured cells and δ-lysin small zone of lysis with sharp margin.

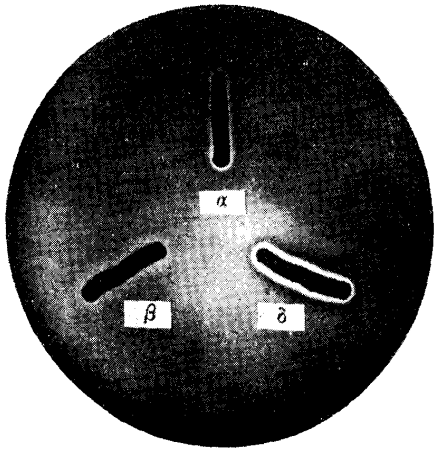


Fig. 3. The mode on human blood agar. α-lysin shows small zone of partial lysis, β-lysin nil and δ-lysin moderately large zone of lysis with sharp margin.