A STUDY OF STREPTOCOCCI PRODUCING POSITIVE HOTIS REACTIONS

ERNEST C. McCULLOCH AND STEWART A. FULLER

Division of Veterinary Science, Agricultural Experiment Station, State College of Washington, Pullman, Washington

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The Hotis test, described by Hotis and Miller (1936) as a simple method for detecting mastitis streptococci in milk, is attractive to veterinarians and milk control officials because the technic is relatively simple and positive tests are easily read. To 0.5 ml. of a 0.5 per cent sterile, aqueous solution of the indicator dye, brom-cresol-purple, in a sterile test tube is added 9.5 ml. of the milk, and this is incubated for 24 hours at 37.5°C. According to the originators of the test, "If streptococci are present, the color changes from purple to a yellow shade during incubation as a result of the production of acid from lactose by these organisms. In addition to this change, if S. agalactiae is present, small flakes or balls of growth, from 0.5 to 4 mm. in diameter, usually form on the side of the tube."

Hotis had earlier noted a tendency of Streptococcus agalactiae to grow in clumps, and Devereux (1935) wrote, "In making brom thymol blue keeping-quality determinations of milk on quarter, cow samples, and producer samples, it was noted that a flocculent growth frequently occurred. This was typical of the type of growth often produced by streptococci. Microscopic examinations verified these findings in that either short or long chain streptococci were present in large numbers when this type of growth was noted. However, there was no apparent difference in the growth produced by Streptococcus lactis and the mastitis organisms... It was also found that only about half of the..."
samples that were culturally positive for mastitis streptococci displayed this flocculent growth.

COMPARISON OF THE HOTIS TEST WITH OTHER TESTS FOR MASTITIS

Hotis and Miller found the Hotis test to be in perfect agreement with the blood-agar method in 715 of a series of 753 samples, and they obtained mastitis streptococci on blood-agar plates from every one of the 560 samples which gave flakes and typical color changes. This led them to conclude that, "A characteristic change in the color of the sample after incubation together with the occurrence of flakes or balls of growth indicates the presence of S. agalactiae."

<table>
<thead>
<tr>
<th>NUMBER OF QUARTERS WITH</th>
<th>NUMBER OF SAME QUARTERS WITH</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH over 6.8..............</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Catalase over 3 ml. gas</td>
<td>27</td>
</tr>
<tr>
<td>Chlorides over 0.1595 per cent</td>
<td>41</td>
</tr>
<tr>
<td>Hotis + reaction</td>
<td>4</td>
</tr>
</tbody>
</table>

Bryan and Devereux (1937) compared the results obtained by direct microscopic examination, blood-agar plates, and the Hotis test. The blood agar detected 89 per cent of the composite samples positive to the microscopic test as compared with 92.5 per cent when quarter samples were tested. Considering the results of the microscopic test as accurate in detecting the presence of mastitis streptococci, they found the Hotis test, after 24 hours incubation, detected from 52.8 to 64.3 per cent of the positive composite samples; after 48 hours, from 62.2 to 71.4 per cent. The correlation was somewhat better with quarter samples, being 64.3 per cent after 24 hours and 78.6 per cent after 48 hours incubation. However, the longer period of incubation produced so many suspicious reactions that the results were difficult to interpret.
When studies were made of quarter samples from the dairy herd of the State College of Washington at Pullman, the results of the Hotis test did not correlate with those obtained by determining the pH either electrometrically or colorimetrically, the catalase test basing a positive reaction on the formation of three or more cubic centimeters of gas, or the chloride test using 0.1595 per cent of chlorides as the maximum in normal milk. All but four Hotis tests were negative, although clinical mastitis occurs in this herd with moderate frequency.

Quarter samples from the herd of the Western Washington Experiment Station at Puyallup revealed a larger percentage of Hotis positives, although the incidence of clinical mastitis in this herd is low.

PURE CULTURE STUDIES OF HOTIS-POSITIVE AND HOTIS-NEGATIVE QUARTERS

In order to ascertain whether or not the positive Hotis tests obtained were due to the presence of *Streptococcus agalactiae* and whether this organism was present in quarters yielding abnormal milk but which gave negative Hotis reactions, studies were made of pure cultures from both normal and abnormal quarters yielding positive Hotis tests and from abnormal quarters yielding negative Hotis tests. The cultures were obtained by streaking fresh quarter samples, incubated quarter samples, or the flakes from typically positive Hotis tests on blood agar plates and picking typical colonies into litmus milk or glucose veal-infusion broth. To ascertain whether or not the pure cultures so obtained would produce typically positive Hotis reactions when grown in a suitable environment, they were cultured in aseptically-drawn brom-cresol-purple milk. This milk, obtained from quarters consistently negative to the Hotis reaction, consistently gave typical Hotis reactions when inoculated with a known strain of *Streptococcus agalactiae*.

The use of unsterilized milk, however, as a culture medium for these studies was recognized as unsound since aseptically-drawn milk rarely is sterile. The necessity of avoiding the use of unsterilized milk was further emphasized by Hucker (1937) who
reported that, "A study of twenty-four udders aseptically re-
moved and cultured from cows known to be free of mastitis and
to have passed through one or more lactation periods shows that
all contained mastitis streptococci."

When autoclaved milk was substituted for the raw milk, only
an occasional tube had the typical flakes on the side, although the
organisms grew readily and produced sufficient acid to alter the
color of the indicator. Varying the concentration of the in-
oculum, changing the dye concentration, adjusting the pH to
6.8, 7.0, or 7.2 with disodium acid phosphate, raising the chloride
content to a total chloride concentration of 0.16 per cent, or
adding calcium to compensate for possible precipitation during
 sterilization failed to enable the milk to support a positive Hotis
reaction. Symbiotic relationships between various milk organ-
isms were improbable because, when typical flakes from positive
reactions were transferred to autoclaved brom-cresol-purple
milk, they failed to give positive Hotis reactions.

The altered chemical composition of mastitic milk is due prin-
cipally to infiltration of blood elements through the injured
mammary tissue. From 10 to 15 per cent of blood serum from
either equines or bovines therefore was added to the autoclaved
brom-cresol-purple milk to raise the pH to approximately 7.0.
Inoculation with a known Hotis-positive strain of streptococci
and incubation for 24 hours produced typical flakes and color
changes in every case.

It was evident, therefore, that heating inactivated or destroyed
some factor in fresh milk which was responsible for the tendency
certain streptococci to grow in flakes on the side of a test tube
and that blood serum either reactivated the milk or supplied
that which had been destroyed.

THE RÔLE OF AGGLUTININS IN THE HOTIS REACTION

Normal milk which did not give a Hotis reaction but which
supported the Hotis reaction when inoculated with a certain
strain of streptococci, was divided into two parts. To both parts
was added 0.025 per cent brom-cresol-purple and to one was
added 20 per cent equine blood serum known to be capable of
activating the Hotis reaction with the strain of streptococci used in this experiment. Portions of each were subjected to various degrees of heat for different periods of time. After cooling, they were inoculated with a strain of streptococci known to produce large flakes in the Hotis reaction and were incubated.

Table 2, the composite of three trials, shows that exposure to 80°C. for five minutes destroys, and exposure to 70°C. for approximately 30 minutes weakens the ability of milk or milk-serum mixtures to support the Hotis reaction. This is within the range of time and temperature which inactivates agglutinins. According to Jordan, "Agglutinin is weakened by heating to from 60 to 70°C. and is destroyed at 75°C. With serum heated to 78°C., no agglutination appears." It also will be noted that milk-serum mixtures were slightly more thermo-tolerant than milk alone, probably because the heat destruction of agglutinins is a gradual process and the serum supplied a greater initial amount.

The more intense reaction in milk and milk-serum mixtures heated to 60° than to 50°C. probably is due to the destruction of the complement and the inactivation of the bactericidal properties of the blood serum and milk at 60°C. The streptococci studied uniformly grew more rapidly in heated than in fresh milk and grew least rapidly in fresh milk to which blood serum had been added.

Early bacteriologists recognized the tendency of streptococci, pneumococci, and certain other organisms to grow in clumps when the medium contained agglutinins specific for them. The reaction was first reported by Charrin and Roger (1889) with Pseudomonas pyocyaneus, and Pfaundler (1898) made similar observations with Escherichia coli and Proteus vulgaris. This phenomenon has been used for the identification of the pneumococci which are made to grow in long threads, often arranged in tangled masses. The process is essentially one of agglutination during growth. In describing this phenomenon, Arkwright (1931) wrote, "An appearance seen in young cultures grown in specific immune serum diluted with broth, which is known as the thread reaction, is almost certainly due to the same causes as
<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>NORMAL MILK</th>
<th>NORMAL MILK PLUS 20 PER CENT SERUM</th>
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<tbody>
<tr>
<td></td>
<td>5 10 15 20 30 60</td>
<td>5 10 15 20 30 60</td>
</tr>
</tbody>
</table>

Not heated........ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++

50°C.............. ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++

60°C.............. +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++

70°C.............. +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++

80°C.............. - - - - - - - - - - - - - -

--- no flakes on the side of the tube.

+, one large flake or from one to five small flakes on the side of the tube.

++, two to five large flakes or five to ten small flakes on the side of the tube.

++++, five to ten large flakes or numerous small flakes on the side of the tube.

+++++, very numerous large or small flakes on the side of the tube.
produce somatic or capsular agglutination, acting during the growth and division of the bacteria in the presence of agglutinins.” The same alteration of the surface which causes bacteria to clump would tend to make them migrate toward any interface, in the Hotis test the wall of the tube, and adhere.

Further evidence that agglutinins are responsible for the characteristic flake formation on the side of the Hotis tube is seen in the fact that the titre of any serum for activating the Hotis reaction could be markedly reduced against any one serological group by agglutinin absorption methods.

Spontaneous agglutination of many strains of organisms has long been recognized. Some strains of *Streptococcus agalactiae* used in this work occasionally produced flakes on the side of the tube of autoclaved brom-cresol-purple milk when incubated without serum. Recently, a beta-hemolytic streptococcus isolated from a case of scarlet fever grew in flakes, typical of those of the Hotis test, on the side of a tube of glucose broth. The work done in this experiment, however, shows that only rarely are the forces responsible for spontaneous agglutination sufficient to give rise to the formation of flakes or balls on the side of a tube of autoclaved milk.

DISTRIBUTION OF HOTIS-ACTIVATING AGGLUTININS

Agglutinins capable of activating the Hotis reaction appear to be widespread. Blood sera from over 90 per cent of the Hotis-negative cows, from all of the Hotis-positive cows studied, from all of several horses which served as sources of blood, from all of six cavies, two humans and one chicken were capable of supporting typical flake formation when added to autoclaved brom-cresol-purple milk and inoculated with *Streptococcus agalactiae* or other streptococci capable of causing typical reactions.

In the herd of the State College of Washington, all quarter samples from 18 Hotis-negative cows supported the Hotis reaction when inoculated with a pure culture of streptococci obtained from a positive Hotis reaction. None of the uninoculated controls gave a positive reaction nor did the culture when inoculated into autoclaved brom-cresol-purple milk.
However, aseptically drawn unheated milk from an experimental herd free from any known carriers of udder streptococci, failed to support the Hotis reaction when inoculated with \textit{Streptococcus agalactiae}. When human blood serum was added at the time of inoculation, typical reactions developed after incubation. Presumably, the milk from the animals in this experimental herd was free from agglutinins for this group of streptococci.\textsuperscript{2}

**DISTRIBUTION OF HOTIS-POSITIVE STREPTOCOCI**

The widespread distribution of Hotis-activating agglutinins suggested an equally wide distribution of Hotis-positive streptococci. Throat swabs were taken from 155 students and inoculated into sterilized brom-cresol-purple milk plus ten per cent of either equine or human blood serum and incubated 18 to 24 hours. Of these, 140 gave typical positive Hotis reactions, 12 were negative, and three questionable. All contained streptococci and when streaked on blood agar and incubated, yielded weakly hemolytic colonies which could not be distinguished from those of \textit{Streptococcus agalactiae}. These data indicate that streptococci from most human throats will produce the Hotis reaction when grown in the presence of human or equine blood serum.

Further trials were made to ascertain whether streptococci from human throats would produce typical Hotis-positive reactions when grown in unheated milk. Twelve of the throat swabs which yielded positive results in the previous experiment were used to inoculate normal, Hotis-negative milk from a single quarter. In the absence of added blood serum, four of these yielded positive Hotis reactions. This would indicate that this apparently normal quarter produced milk which contained agglutinins for streptococci from at least four of twelve human throats.

In another trial, milk samples from seven apparently normal Hotis-negative quarters were inoculated with throat swabs from one person and five of these after incubation gave positive Hotis reactions.

\textsuperscript{2} The authors wish to express their appreciation to Dr. O. W. Schalm of the University of California for his cooperation in this portion of the experiment.
Fifteen nasal swabs from Hotis-negative cows yielded two Hotis-positives when inoculated into autoclaved milk plus serum. Microscopically and on blood agar these organisms had the appearance of *Streptococcus agalactiae* and they hydrolyzed sodium hippurate and failed to utilize esculin.

Vaginal swabs from 11 Hotis-negative cows and tears from four Hotis-negative cows did not give positive Hotis reactions when inoculated into autoclaved milk plus serum.

**CULTURAL CHARACTERISTICS OF HOTIS-POSITIVE STREPTOCOCCI**

Using a capillary pipette, typical flakes were fished from the side of Hotis-positive tubes into 2 ml. of glucose broth. After four hours incubation, the cultures were streaked on five per cent bovine blood agar and incubated over night at 37.5°C. Typical colonies were transferred to glucose broth and these incubated for 24 hours; then loop transfers were made into various media. The diversity of action of these streptococci on carbohydrates is shown in table 3.

The bovine strains most frequently isolated utilized sodium hippurate but not esculin. The strains from human sources, in general, utilized neither. In both types, however, there were variations, including human strains, that had the same reactions as *Streptococcus agalactiae*; i.e., utilization of sodium hippurate but not esculin.

Thirty-seven cultures of streptococci isolated from cows and 61 isolated from human beings were identified serologically using the Lancefield technique as modified by Brown and in addition were grown in esculin and sodium hippurate broths, and in sterilized brom-cresol-purple milk plus ten per cent serum. Most human cultures were grown in the same serum which was used for the original Hotis test, but many of these did not produce typical flakes in pure culture until grown in a different serum-milk mixture. Not all pure cultures of bovine origin produced positive reactions in any one serum.

From table 4 it will be seen that most of the bovine strains isolated from flakes in positive Hotis tubes belong to Lancefield's serological group "B," and that all but five of these produced a
TABLE 3

Carbohydrate reactions of Hotis-positive streptococci

<table>
<thead>
<tr>
<th></th>
<th>Sodium hippurate</th>
<th>Esculin</th>
<th>Salicin</th>
<th>Mannitol</th>
<th>Inulin</th>
<th>Arabinose</th>
<th>Raffinose</th>
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<tbody>
<tr>
<td>Strains from bovine sources</td>
<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>Strains from human sources</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+, hydrolysis; -, no hydrolysis.

TABLE 4

Serological grouping of streptococci

<table>
<thead>
<tr>
<th>BOVINE STRAINS</th>
<th>HUMAN STRAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains</td>
<td>Group</td>
</tr>
<tr>
<td>20</td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
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<tr>
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<td>B</td>
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<tr>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>?</td>
</tr>
</tbody>
</table>

Group = Lancefield's serological group; ? = not in Lancefield's serological groups "A," "B," or "C"; + = positive Hotis reaction in pure culture; H = hydrolysis; ac = acid.

positive Hotis reaction in pure culture when grown in the modified medium.
STREPTOCOCCI PRODUCING HOTIS REACTIONS

Of the human strains isolated from flakes in positive Hotis reactions from throat swabs in the modified medium, seven belonged to Lancefield's group "B," one to group "A," and fifty-three to neither "A," "B," or "C"; twenty-one of these belonging to some other than the first three groups, yielded positive Hotis reactions in pure culture when grown in the modified medium using equine serum.

As noted above, some of the cultures would not form flakes when grown in the same serum used in the original test, but would in another serum. Furthermore, any one culture might not form typical flakes in every one of a series of tubes using one serum, although clumps would appear in the bottom of the tube. This would suggest the presence of several groups of agglutinins and also that the migration of streptococci to the sides of the tube when grown in the presence of agglutinins specific for them is somewhat a matter of chance. Some strains, especially those which showed some tendency towards self-agglutination, produced typical flakes with more frequency than did certain other strains. The presence of several groups of agglutinins and perhaps the matter of chance in the migration to the sides of the tube is further suggested by the results obtained in the previously-mentioned series of throat swabs in normal milk without added serum.

DISCUSSION

The Hotis test appears not to be specific for *Streptococcus agalactiae*. Even when attempts were made to confirm the identity of the organisms by streaking Hotis-positive milk on blood agar and selecting only the weakly hemolytic colonies for further study, many diverse streptococci were included. It is evident that the combined use of the Hotis test and blood agar plates is not sufficient for the differential diagnosis of *Streptococcus agalactiae*.

The presence of weakly hemolytic, Hotis-positive streptococci in most human throats and in the nasal discharges of some Hotis-negative cows throws serious doubt on the assumption that the elimination from a milking herd of every cow that is
sheddung such streptococci in the milk will result in the herd remaining free from streptococcic infection. Apparently, streptococci of this general class are much more ubiquitous than formerly supposed.

In herds such as were available to the originators of the Hotis test, in which the organism most frequently associated with mastitis is *Streptococcus agalactiae*, the Hotis test may continue to have some value. In other herds, the results may be confusing or misleading. The identification of an organism by means of an agglutination reaction, in which both the organism and the agglutinating serum are obtained from the same clinical case, must be recognized as scientifically questionable.

**SUMMARY AND CONCLUSIONS**

The Hotis test has been described as being specific for *Streptococcus agalactiae*, but in this work, using both unheated milk and sterilized Hotis-negative milk plus blood serum, it has been found that many organisms, of the genus *Streptococcus* at least, are able to produce the reaction.

The Hotis reaction appears to be an agglutination reaction and the alteration of the surface which causes the organisms to clump would cause them to tend to migrate to the surface of the test tube and adhere.

It appears that the yellow flakes or balls which form on the side of a tube of brom-cresol-purple milk after incubation, and which have been considered diagnostic of *Streptococcus agalactiae* in the Hotis test, may be produced by any organism which:

1. Stimulates the production of agglutinins.
2. Grows in the presence of 0.025 per cent brom-cresol-purple in a milk medium when incubated in a test tube under aerobic conditions at 37.5°C.
3. Forms clumps on the side of the tube when grown in the presence of its agglutinins.
4. Produces sufficient acid from lactose to increase the hydrogen ion concentration to about pH 5.4.
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