The Rapid Identification of *Streptococcus agalactiae*

Alfred H. Brueckner and Stanley E. Hartsell, Purdue University

The typing of streptococci by serological methods, particularly the precipitin technique of Lancefield (1928) has been shown to be reliable not only for the identification of cultures but also for the demonstration of the various antigenic constituents of their cells. To those interested in the rapid detection of streptococci the problem of making this technique available to dairy and health laboratories is receiving considerable attention at this time.

It is obvious that such a method would be of great assistance to these institutions; however, one feature of precipitin techniques is that the preparation of large numbers of extractives from the cultures and the necessary centrifugation of the cells is time-consuming. These factors may be a limitation in some laboratories doing routine bacteriological work with milk, and any test which could obviate these difficulties would be greatly appreciated.

It appears that a rapid, inexpensive, and reliable method for the determination of Group B streptococci by the agglutination method could be used to good advantage in reducing the number of cultures which would require the precipitin technique before their group would be known. However, we observe frequently that any attempt to utilize agglutination methods with cultures of streptococci has had little success because of the tendency of these forms to agglutinate spontaneously. Griffith (1926) devised a method with which he could classify *Streptococcus pyogenes* by the use of a rapid slide agglutination test. Later Stableforth (1932) utilized a similar technique for the identification of *Streptococcus agalactiae*. In a recent report Stableforth (1938) records the extensive use of this test in typing many strains of this microörganism. Even the identification of sub-types within Group B streptococci was accomplished with relative ease. Some cultures were spontaneously agglutinable or inagglutinable, and further tests were required before their serological type could be known. Plastridge and Hartsell (1937), in their work on the biochemical and serological reaction of *Streptococcus agalactiae*, discarded this method because of these difficulties.

The present report describes the development of a microscopic slide agglutination technique for the rapid detection of *Streptococcus agalactiae* after its isolation from routine milk samples. Spontaneous agglutination among cultures has been greatly reduced, and by the use of this test *Streptococcus agalactiae* can be identified in 72 hours after the milk sample has been taken. No biochemical or involved serological tests are required to type most of the strains of this organism. An attempt to adapt the Neufeld technique to the identification of *Streptococcus agalactiae* will also be discussed.

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Preliminary Experiments

In studying the biochemical and serological relationship of *Streptococcus agalactiae* (1937), the encapsulation of some cultures was noted on primary isolation. Stained smears made from the incubated milk tubes showed well marked capsules; however, these structures were seen so infrequently that they were regarded as artifacts. It seemed appropriate to reinvestigate this problem with a view to adapting the Neufeld technique for the typing of pneumococci to the identification of *Streptococcus agalactiae.* Such an adaption, if successful, would obviate the necessity of using blood agar plates and subsequent biochemical and serological determinations of the pure culture for the detection of this species in milk.

Cultures.—Twenty-four cultures of streptococci, regarded as members of Lancefield's Group B, were received from Dr. W. N. Plastridge. These strains included the well known cultures "090 T" and "V9" of Lancefield, as well as strain PH 281, which was isolated originally by Mr. Mickle. The first two of these strains have been studied extensively by many investigators, the most recent report concerning their encapsulation being that of Dawson (1938).

Antibacterial serums.—Antiserums were prepared by injecting rabbits intravenously with a 0.5% suspension of packed formalized cells in saline solution. These antigens contained the growth from cultures cultivated in a proteose-peptone, phosphate beef-infusion medium for 48 hours. The rabbits were injected according to the method of Foote, Welch, West, and Borman (1936), which had been shown to be suitable for the production of satisfactory Group B antiserum (1937).

Capsule stimulation.—Since most of the pure cultures did not show a well-defined capsule, the first problem was that of inducing the development of this structure before the Neufeld technique could be applied. Daily transfers of the 24 strains were carried out in a blood-milk broth with a beef-infusion base. Examinations for capsules by the method of Howie and Kirkpatrick (1934) after three transfers showed slight capsule formation in 13 of the 24 strains. It was observed that capsular development under these conditions was variable and that large capsules were never found.

Negative results were also obtained by using blood-milk agar slants (5% cow blood, 5% milk in beef infusion agar) and a skimmed milk medium even when daily transfers were continued for ten days. Passage of the cultures through mice failed to stimulate a definite increase in capsular material. In the presence of homologous serum, 090 T and all other strains failed to show capsule swelling.

In view of the work of Jennings (1931) with human streptococci, the methods of bacterial variation were employed to encourage capsule formation. He showed that unencapsulated streptococci might become capsulated and grow as mucoid colonies on blood agar after many transfers in blood media. Dawson (1938) has recently reviewed the litera-

ture on this subject and records the development of the mucoid phase and capsules for strain 090 T.

It was thought that an encapsulated strain of *Streptococcus agalactiae* (possibly strain 090 T) might be used with the serum of cows to detect mastitis. Then it would be possible to eliminate the use of any cultural methods for the detection of infection due to this species. The presence of antibodies in the serum of the infected cow would constitute a positive diagnosis. With this viewpoint in mind strains 090 T, V9, and PH 281 were grown in blood broth and transferred daily for six days. On the seventh day blood agar plates were streaked and then incubated for 24 hours at 37° C. A small inoculum from the edge of the colony was transferred again to blood broth. This procedure was carried through six series without a mucoid phase being observed in any of the plates. This part of the work is being extended.

Observation of capsule swelling.—Whenever even slightly encapsulated organisms were noted in the above experiments, they were subjected to exposure to homologous serum in a hanging drop and observed for capsule swelling. The first hanging drops were examined immediately as in the Neufeld technic. Then incubation of the serum-antigen mixtures was tried at 37° C. for one hour. Even when these preparations were held at this temperature for 24 hours in a humid atmosphere (water bath), no capsule swelling was seen. In these experiments we noted that many of the serums caused a consistent and definite agglutination of their antigen; and in view of the many unsuccessful attempts to produce the swelling, a further study of this agglutination was considered expedient.

The Microscopic Agglutination Test

The same serums used in the work just reported were employed in a reinvestigation of the microscopic agglutination test. It was first demonstrated that serums for strains 090 T, V9, and PH 281 had a precipitin titre of 1-100 and that only a slight reaction was observed in cross-precipitin tests. The precipitation with the homologous serum was sufficiently strong so that there was no question as to type relationships. Cross-agglutination tests also supported these correlations. In the identification of *Streptococcus agalactiae* on primary isolation the three antiserums for Lancefield's types I (Strain 090 T), II (Strain V9), and III (Strain PH 281) were employed.

Strains to be used as antigens were grown in beef-infusion proteose-peptone broth containing approximately 5% defibrinated cow blood. One loopful of the broth culture which had been incubated at 37° C. for 12-18 hours was removed from the upper half of the medium and placed on a clean cover slip. Every effort was made to keep from agitating the culture, since very few calls were needed in the hanging drop; and large granular clumps of cells which occur frequently on the sides of the tube would confuse the detection of agglutination. One loopful of undiluted antiserum was then added to the drop of culture. The addition of enough alkaline methylene blue to this mixture to color it lightly facilitates reading the tests but does not alter the reaction. The coverslip was

mounted in the usual way and the preparation incubated over water at 45-50° C. in a water bath. Readings were made by microscopic examination of the hanging drop with the high dry lens. Final readings were made after four hours' incubation, although most positive tests were detected in two hours. A control, containing only a loopful of the broth culture, was made for each organism so that spontaneous clumping, if it occurred, would be detected. Positive tests showed definite clumping of the organisms as contrasted to the free chains of cells in the control. An arbitrary system of recording the extent of agglutination was adopted: 3+, approximately 90% of the cells in the hanging drop were in aggregates; 2+, about 50% were in clumps; 1+, from 10 to 20% of the cells showed agglutination. Occasionally, clumping reached a macroscopic state (4+) in strongly positive tests, and the clumps could be seen in the drop with the unaided eye.

Experimental

If the microscopic agglutination test is to be of any value in the routine examination of milk samples, it should require a minimum of time and materials and still give reliable results. With this viewpoint in mind, 261 quarter-samples of 59 cows were taken in two different herds and the 75 isolations of streptococci examined by the above technique.

Procedure with isolated strains.—The method of Plastridge and his associates (1934) was used in collecting the milk samples aseptically. They were incubated over night at 37° C. and then examined culturally. One loopful of each incubated sample was streaked on one quarter of a cow-blood agar plate and then incubated for 24 hours at 37° C. Breed smears were also made and examined for the presence of streptococci and for leucocytes in the incubated milk. All weakly hemolytic colonies were picked from the plates and transferred to beef infusion blood broth. In a few cases short chain streptococci were seen in the microscopic examination, but no hemolytic colonies were found on the original or repeated platings. These cultures proved to be micrococci or streptococci, giving biochemical reactions typical of *Streptococcus lactis*. All isolations in the present investigation which gave typical *Streptococcus agalactiae* biochemical reactions were weakly hemolytic. The broth cultures obtained from the blood agar plate colonies were used as antigens in the microscopic agglutination test. Each isolation was tested with antiserums prepared against organisms representing the three types of Lancefield's Group B Streptococci. Biochemical studies of all isolations were made according to the method of Plastridge (1934).

Results

Preliminary isolation experiments were done with a local dairy herd which was at that time having trouble with mastitis. Encouraging results were obtained with the microscopic slide agglutination tests, and a more comprehensive survey of a larger herd was undertaken to determine the efficiency of the test. Table I shows the biochemical and serological reactions of certain isolated strains.

TABLE I.—Identification of Selected Isolated Strains of Streptococcus agalactiae

Culture Cow Number	Source Quarter	Biochemical Reactions									Microscopic Slide Test Type Serums			
		Hemolysis	L.M.	M.B.	L.	Mn.	I.	Raf.	Sal.	Ar.	I (090 T)	II (V9)	III (PH 281)	Control
302	L.F.	beta	ACpR	0	A	0	0	0	A	0	0	2+	3+	—
295	R.R.	beta	ACpR	0	A	0	0	0	A	0	3+	3+	2+	—
299	L.R.	beta	ACpR	0	A	0	0	0	Sl.A	0	2+	2+	3+	—
290	L.F.	beta	ACpR	0	A	0	0	0	A	0	2+	2+	+	±
521	L.R.	beta	ACpR	0	A	0	0	0	A	0	+	3+	3+	—
306	L.F.	beta	ACpR	0	A	0	0	0	A	0	3+	3+	3+	—

Abbreviations: L.F.=left front quarter; L.R.=left rear quarter; R.R.=right rear quarter.
Biochemical Reactions: in Litmus milk, ACpR=acid, coagulation and partial reduction. L.=Lactose; Mn.=Mannite; I=Inulin; Raf.=Raffinose; Sal.=salicin; Ar.=Arabinose.
Reactions in Methylene Blue Milk: 0=no reduction.
Fermentation Reactions: A=acid; 0=no acid.
Serological Reactions: 0=no agglutination; +=approximately 20% of cells agglutinated; 2+=approximately 50% of cells agglutinated; 3+=approximately 90% of cells agglutinated.

It will be noted that there is a remarkable agreement between the biochemical and serological properties of these strains. Seventy of the 75 isolations made in the present investigation gave cultural and biochemical tests which identified them definitely as *Streptococcus agalactiae*. As stated previously, only the reactions of representative isolations were included in the table. The other five strains gave reactions which were atypical of *Streptococcus agalactiae*, notably the complete reduction of methylene blue and litmus milk. These five cultures gave negative serological reactions, except for one, which gave a slight reaction with 090 T antiserum.

Of the 70 strains classified as *Streptococcus agalactiae*, 69 or 98.5% were detectable by the microscopic agglutination test, the one exceptional strain being spontaneously agglutinable to the point where clear-cut readings could not be made. Forty-four of the 70 *Streptococcus agalactiae* strains were isolated from different quarters, and 26 were obtained in repeat tests on the same quarters. Since the strains isolated on the second sampling of a given quarter often gave strong evidence of being the same as those isolated in the original sampling, especially in the intensity of the agglutination reaction; it is of interest to survey the data from this standpoint. In Table II we note that practically all strains, when isolated from the first milk sample of a quarter, can be designated easily as *Streptococcus agalactiae*. Only one of the 44 strains could not be typed. This indicated definitely that the test was

TABLE II.—Degree of Reaction of Isolated Strains Against All the Test Serums

Degree of Reactions*	Original and Repeat Isolations		Original Isolations Only	
	No. of Strains	% of Total Strains	No. of Strains	% of Total Strains
Strongly Positive	37	52.9	29	65.9
Positive	24	34.3	13	29.5
Weakly Positive	8	11.4	1	2.3
Spontaneous	1	1.4	1	2.3
Total	70	100.0	44	100.0

*The arbitrary standards for degree of reaction:
Strongly Positive = 3+ or 4+ reaction with at least one of the three test sera.
Positive = 2+ reaction with at least one serum and some reaction with two sera.
Weakly Positive = 1+ reaction only with any serum.
Spontaneous = cells agglutinated even in absence of any sera.

of value when a rapid determination for Group B streptococci was desired.

The question arises as to whether the three test serums employed in our investigation need be used when testing routinely for *Streptococcus agalactiae* or Group B streptococci in general. In this connection it should be remembered that, while the serums were not wholly type specific, they reacted much more strongly toward homologous than toward heterologous organisms. A survey of the weak and negative reactions of the 44 strains of *Streptococcus agalactiae* isolated from different quarters (Table III) shows that in our work it was advisable to use the three test sera.

Stableforth (1938) has shown that there is rather a close correlation between the infecting types of *Streptococcus agalactiae* in the different quarters of a cow and the prevalence of these types in the herd. The spread of the organism from one quarter to another quarter in the same cow has been shown by agglutination methods to be brought about by the

TABLE III.—The Number of Strains Giving Negative or Weak Reactions with Each Test Serum

Antiserum Prepared Against Strain:	Negative No.	Strains %	*± and + strains		Total % —and Weak
			No.	%	
090 T	2	4.6	8	18.6	23.2
V 9	1	2.3	4	9.3	11.6
PH 281	2	4.6	7	16.3	20.9

*4 strains weak with both 090 T and PH 281; 1 strain weak with both 090 T and V9.

same serological types. Some evidence to support Stableforth's observation was noted in the use of the microscopic agglutination test. Because of cross reactions found in the preliminary titrations, little was expected in the way of typing organisms. However, it was soon obvious that the isolated strains did show differences in their reactions toward the three sera, and typing of a sort was taking place.

The strains of *Streptococcus agalactiae* from the different quarters of 7 of 13 cows gave identical agglutination reactions. Five of the 13 cattle were re-tested a second time, and the data indicated that the infecting types did not change during the interval (three months) between tests. It is realized that the above data are too few to support all the facts on the spread of mastitis infection; however, the possibilities of the microscopic agglutination test in such a study is indicated clearly.

Agglutination tests were also made with all sera against the following species of streptococci: *Streptococcus lactis* (5 strains), *Streptococcus bovis* (4 strains), *Streptococcus citrovorus* (1 strain), *Streptococcus scarlatinae* (1 strain), *Streptococcus epidemicus* (1 strain), *Streptococcus fecalis* (2 strains). We are indebted to J. M. Sherman, Alice Evans, and B. W. Hammer for subcultures of these species. One strain of *Streptococcus bovis* (No. 113) gave a slight reaction in the presence of V9 antiserum. Three strains of *Streptococcus lactis* and one strain of *Streptococcus fecalis* reacted spontaneously; otherwise, all other agglutination tests were negative.

Discussion

From our results it is evident that the microscopic agglutination test can be used successfully in the identification of *Streptococcus agalactiae*. The difficulties of spontaneous agglutination have been to a large extent minimized by the use of young serum-broth cultures as antigens. Apparently, the serum present in the blood broth sensitizes the cells to the action of the agglutinins, thus reducing the probability of agglutination due to the electrolyte in the absence of specific antiserum. Since cow blood was used in preparing the broth media in this investigation, the possible influence of antibodies in the blood from a mastitis-infected cow was considered. We used the blood from cows shedding *Streptococcus agalactiae* and from other animals which were free from this infection. No difference was discernable in intensity of the agglutination reaction or in the type specificity of the streptococci when blood from these sources was employed in the preparation of media.

The use of a small amount of the broth culture, which is free from clumps of cells often observed in cultures of streptococci, may also minimize the probability of spontaneous agglutination. The necessity of resuspending the cells in a solution containing an electrolyte has been obviated and may account, in part, for the detection of 98% of the cultures without this limitation. The centrifugation of broth cultures has been eliminated in the slide agglutination test. Concentrated cell suspensions are not only unnecessary but even detrimental in the microscopic agglutination test.

It is of interest to compare the microscopic agglutination test and

the rapid macroscopic agglutination test of Stableforth (1932). In the latter method "suspected colonies are picked into 30 cc. of serum broth, each streptococcal growth is centrifuged, and the deposit taken up in 0.5-1.0 cc. cabol-saline. A slide agglutination test occupying one minute is then made with the five sera, representing most of the British strains. A definite reaction with any of these is indicative of the particular type. If the result is negative, cultures are prepared in 100 cc. of glucose broth, extracted, and tested by the contact method with a group serum known to be specific for *Streptococcus agalactiae* or Streptococcus Group B."

In Stableforth's test the difficulty of spontaneous agglutination has not been satisfactorily overcome, since it is this phenomenon which limits observations to a one-minute period. In this one-minute observation time weakly agglutinable strains are evidently missed, and a retest is necessary. In the microscopic agglutination tests the suspensions are stable, with very few exceptions; and weakly agglutinable strains can be appropriately identified. The incubation period is longer, obviously; but confirmatory or repeat tests are unnecessary. This accounts for much of the reduction in time when typing large numbers of strains.

The quantity of medium required in the test is another consideration which should be recalled. The rapid macroscopic agglutination test and the precipitin test of Lancefield require a minimum of 30 cc. of medium. As little as 1 cc. of blood-broth culture will provide sufficient antigen for the microscopic agglutination tests.

Cross tests with streptococci belonging to groups other than Group B have shown the specificity of the test, and biochemical confirmation with all isolated strains has served to prove its sensitivity.

It is possible that the test described here, in addition to its practical applications, might have taxonomical uses if different immunological methods were utilized and absorbed sera employed in the test. According to Lancefield (1934), specific type antibodies must be made by several series of injections with the group specific antibodies giving way to the type specific antibodies as the injections progress. Stableforth in his agglutination test for types used absorbed serums. However, in the detection of *Streptococcus agalactiae* as a species, serums of high group titre are preferable for the identification of all Group B strains isolated. Though a knowledge of the various subtypes of Group B is of significance to those interested in the epidemiology of mastitis infection, the classification of an isolated strain as a Group B microörganism would probably be sufficient from the standpoint of dairy and health laboratories. The practicability of the microscopic agglutination test in this regard is evident.

Conclusions

An attempt to adapt Neufeld's capsule swelling to Group B streptococci failed for lack of a method of rapidly inducing consistent capsule formation in the species tested.

A rapid microscopic agglutination test for Group B streptococci is presented. The test has shown close correlation with biochemical tests for the identification of *Streptococcus agalactiae.*

The test was successful in identifying 98% of the freshly isolated strains of this microörganism. This efficiency is attributed to the methods employed in obviating the difficulties of spontaneous agglutination which are inherent in cultures of streptococci.

There is a suggestion that data assembled by the use of the microscopic agglutination test is in accordance with the conclusions of Stableforth in his work on the etiology of mastitis.

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