DOUBLE-ZONE BETA-HEMOLYTIC STREPTOCOCCI

Their Cultural Characteristics, Serological Grouping, Occurrence and Pathogenic Significance

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The appearance of double-zone beta-hemolytic streptococci in blood agar has been described in a previous publication (Brown, 1937b). If this striking appearance were one which might be displayed by various hemolytic streptococci it would be nothing more than an interesting phenomenon. Since it is found to be correlated with certain cultural reactions and serological groupings it assumes systematic and diagnostic significance. Its practical value is enhanced because it often enables these streptococci to be recognized in the primary blood-agar plate, even before pure cultures have been obtained.

This report embraces a study of 188 strains, of which 138 are from human sources and 50 of animal origin. Eighty-six strains were received from other workers (R. C. Lancefield, H. Plummer, R. B. Little, G. J. Hucker, L. Kirschner, J. M. Murphy, W. D. Frost, J. M. Rosell, E. I. Parsons). Of these strains 55 were from Lancefield, including 33 isolated by Ronald Hare, 6 by M. H. Dawson and 10 by W. N. Plastridge, the remaining 102 strains were isolated in the author's laboratory.

As indicated in a previous publication (Brown, 1937a), three fermentative groups of double-zone beta-hemolytic streptococci were found with reference to lactose and salicin: lactose−, salicin+; lactose+, salicin+; and lactose+, salicin−. All strains hydrolysed sodium hippurate and fermented glucose, sucrose and trehalose; none fermented mannitol, raffinose, inulin

or sorbitol. The final pH in glucose broth was 4.3 to 4.9; all but three of the strains within 4.4 to 4.6. Eight strains, including representatives of the three fermentative groups, were tested for the fermentation of other substances and all gave the following results: levulose, galactose, maltose, glycogen and dextrin fermented; inositol, adonitol, dulcitol, xylose, arabinose, rhamnose and esculin not fermented.

For the determination of the fermentation reactions of streptococci, three precautions are regarded as essential: the basic medium must be one which supports good growth of the organisms in the absence of fermentation; the test sugars, other than glucose, must not be sterilized in the medium; the cultures must be incubated sufficiently long to detect slow fermentations. In these studies, the basic medium has been broth made from fermented meat infusion to each tube of which 3 or 4 drops of sterile ascitic fluid were added aseptically. The carbohydrates (5 or 10 per cent solutions in distilled water) were sterilized in the autoclave or by filtration and added aseptically to the sterile fermented broth. Before inoculation the media were incubated to determine sterility. The cultures were incubated for at least five days before final readings were made by determining the pH of the glucose broth cultures and noting the color of the brom-cresol-purple which was used as an indicator in the other sugar media. The importance of the above precautions was demonstrated by the study of a number of strains received from other laboratories and reported in the literature as fermenting lactose when tested in Hiss serum water. When these strains were retested by us, they fermented lactose which had been added to Hiss serum water before sterilization, but in Hiss serum water and in fermented infusion broth with lactose added aseptically, there was no fermentation, although good growth occurred.

The hydrolysis of sodium hippurate was determined in infusion broth containing 1 per cent of sodium hippurate. Before testing with ferric chloride reagent, distilled water was added to the previously marked tubes to replace water lost by evaporation during storage and incubation. By observing this precaution, and by using a properly balanced and tested reagent, we have never failed to obtain definite and consistent results.

Twenty-seven strains of double-zone hemolytic streptococci were inoculated onto 40-per cent bile agar. In comparison with

hemolytic streptococci of serological groups A and C most of the strains grew well although a few showed only meagre growth. Our impression is that medium containing 40-per cent bile is not sufficiently differential for final differentiation of the streptococci.

Twenty-one of the double-zone strains of human and bovine origin and representing all of the three fermentative groups were tested for fibrinolytic activity against human plasma by the technique of Tillett and Garner (1935). None was fibrinolytic.

White mice were inoculated intraperitoneally with 0.5 cc. of over-night ascitic-fluid broth cultures of most of the strains. The results are shown in table 1. The mice were observed for a week after inoculation. When death occurred it was usually

TABLE 1

Results of injection into mice

SOURCE	FERMENTATIVE GROUP	MICE	
		Survived	Died
Human	Lactose −, Salicin +	17	61
	Lactose +, Salicin +	15	36
	Lactose +, Salicin −	1	5
Bovine	Lactose +, Salicin +	20	5
	Lactose +, Salicin −	16	1
Guinea pig	Lactose −, Salicin +	1	2
Rabbit	Lactose −, Salicin +		1
Horse	Lactose +, Salicin +	1	

within 48 hours. If the inoculation of mice with such large amounts of culture is of any significance, it would appear that strains of the lactose−, salicin+ group are more frequently pathogenic for mice than are the other strains. It also appears that the human strains of whatever fermentative group are more frequently pathogenic for mice than are the bovine strains.

When hemolysin tests were carried out by adding 0.5 cc. of a 5-per cent suspension of washed rabbit-blood erythrocytes to 0.5 cc. of young serum broth cultures and incubating for two hours, the double-zone hemolytic streptococci were variable in their reactions, i.e., different strains produced various amounts of hemolysis, from slight to complete. Most strains produced

moderate hemolysis. The amount of hemolysis was independent of the source or fermentation reactions of the strains. These streptococci are therefore less consistently hemolytic by this test than are strains of serological groups A and C.

All of the 188 strains of double-zone beta-hemolytic streptococci studied have been found to belong to Lancefield's serological group B by the precipitin test. Seventy-five of these strains had been grouped by other investigators (Lancefield and Plummer) using the Lancefield technique (1933). Most of them, and all of the remaining strains, were also grouped by us, using the microscopic technique of Brown (1938). We have also encountered a few hemolytic streptococci of bovine origin falling into group B which did not produce double zones in our media but which were otherwise culturally like the double-zone strains. It may be said, therefore, that according to our experience to date, all double-zone beta-hemolytic streptococci belong to serological group B but that there may be a few strains of group B which do not produce double zones under the conditions which we have employed.

Sixty-seven of the double-zone strains have been serologically "typed" by Lancefield or Plummer. No close correlation of source, fermentative characters and serological types was apparent. Further study will be necessary to determine whether there is any correlation.

In table 2 the specific sources and the fermentative groups of all of the double-zone beta-hemolytic streptococci studied are listed. In human beings the throat and the genito-urinary tract may be regarded as their habitat. Of the 22 throats from which strains were isolated, only two were described as "sore" and it is not certain that the condition of these two was due to the double-zone streptococci. Nothing is known of two of the tonsil cases and the other two tonsil strains were recovered from removed tonsils, one patient having complained of frequent sore throat. These were the only strains found in the examination of 100 pairs of removed tonsils. Again, the relation of the double-zone streptococcus to the clinical condition is unknown. Most of the 46 vaginal strains were taken post-partum from women with

afebrile puerperium. One was from a woman with a post-partum temperature of 100°F. but without other evidence of infection reported. A few strains were found ante-partum during routine examinations. Of the 32 urine cultures all were from cases of

TABLE 2

Sources and fermentative groups of human strains studied

SOURCE (HUMAN)	LACTOSE− SALICIN+	LACTOSE+ SALICIN+	LACTOSE+ SALICIN−
Throat	7	9	6*
Tonsils	2	2	
Vagina	20	26	
Urine	26	6	
(Probably derived from the mouth or throat)			
Sinus	1		
Lung	10	4	
Heart blood, 15116	1		
Neck abscess		1	
(Probably derived from the genito-urinary tract)			
Pelvic abscess	2		
Blood (abortion) during life		1	
Knee	1		
Heart blood, post-mortem	6		
Peritoneum	2	1	
(Infections of obscure origin)			
Peritoneum		1	
Ventricular fluid (hydrocephalis)	1		
Gangrenous leg	1		
Source unknown (Lancefield 090)		1	

* These six strains were obtained from Plummer and are representative of those found by her in the throats of children in Institution B.

urinary tract infection (cystitis, pyelitis, urethritis, prostatitis). Three-fourths of them were from women. In sixteen instances no other organism than the double-zone beta-hemolytic streptococcus was obtained in culture and the latter was often present in large numbers. There seems no doubt that these streptocci

may be a cause of such infections of the urinary tract. It is to be noted that most of the strains recovered from urine failed to ferment lactose.

Hare and Maxted (1935) were unable to find hemolytic streptococci of serological group B in the stools of 150 women examined. Smith and Sherman (1938) reported six strains isolated from human feces. The normal throat and the vagina of some individuals appear to be the habitats of double-zone beta-hemolytic streptococci. From these sources infection may occur, as indicated in table 2. Some of the cases may be worthy of discussion.

Fourteen strains were obtained from lungs at autopsy but always mixed with other organisms. The pathogenic rôle of the double-zone streptococci is doubtful in these cases.

Case 15116 was one of typhoid fever of several weeks duration (male, age 36). At autopsy there was no perforation of the intestine and no peritonitis but there were found purulent sinusitis, otitis media and a deep ulceration of the vocal cord extended into the cartilage. At the base of the ulcer were masses of Gram + cocci. From the heart blood at autopsy there were obtained large numbers of double-zone hemolytic streptococci and a smaller number of colon bacilli. It seems probable that death was due to terminal blood stream infection by double-zone streptococci derived from the throat.

The pelvic abscesses were in women and in one of them the double-zone streptococcus was found in pure culture.

In the fatal case of septicemia following abortion 100 double-zone hemolytic streptococci per cubic centimeter of blood were found in pure culture during life.

From an arthritis of the knee, double-zone hemolytic streptococci in large numbers were found in pure culture. A similar organism was found in small numbers in the blood stream. There had been a chronic pyuria and the patient was diabetic. Death followed operation on the knee but the cause of death was not determined.

In six other cases double-zone hemolytic streptococci were cultured from the heart blood post-mortem. Three of the cases were infants in the blood of whom only the streptococci were found; one lived only 24 hours after birth, one four days with hemorrhagic disease of the new-born, and one was born prematurely but lived for one month. Infection may have been contracted from the mothers at birth. The

other three cases were adults with pathological findings which suggest possible infection from the genito-urinary tract (carcinoma of the bladder, pyelonephritis and cystitis, arsphenamine poisoning with salpingitis and cysts in the ovaries and cervix uteri).

In three cases peritoneal infection appeared to be correlated with genito-urinary lesions (abscess of the broad ligament, carcinoma of the prostate and adenoma of the kidney, pyelonephritis and cystitis).

In three cases the infections were of obscure origin and in the ventricular fluid of one of them the double-zone streptococci were found in pure culture.

Reference to table 3 shows that 80 of the strains isolated from human beings were of the lactose − group and that 18 of the

TABLE 3

Strains from all sources

SOURCE	LACTOSE− SALICIN+	LACTOSE+ SALICIN+	LACTOSE+ SALICIN−
Human	80	52	6*
Bovine		27	18
Guinea pig	3		
Rabbit	1		
Horse		1	
Totals	84	80	24

* These six strains were obtained from Plummer and are representative of those found by her in the throats of children in Institution B.

strains from cow's milk were of the salicin − group. To date, lactose − strains have not been reported from bovine sources and salicin − strains have not been reported from pathological conditions in man. It is obvious that the human infant may have an opportunity to acquire double-zone hemolytic streptococci at birth. There certainly is no evidence that the lactose − strains found in man are derived from cows milk. Judging by their history (Plummer, 1934) the 6 salicin − strains isolated from children's throats may have been of bovine origin. If the human throat may serve as a carrier of salicin − strains derived from milk, there appears no reason why it may not also carry lactose+, salicin+ strains from milk, but it does not follow that all such strains are

from milk. Strains fermenting both lactose and salicin were from both human and bovine sources and appear to be alike by current methods of study, but to speculate as to whether such strains found in man were originally bovine seems as fruitless as would be an effort to determine whether the colon bacilli originated in the intestinal tract of the cow or man. The pathogenic rôle of these streptococci for man bears a certain similarity to that of the colon bacilli. In the blood stream they may produce septicemia; in the abdomen, peritonitis; in the urinary tract, cystitis or pyelitis; they may infect sinuses or produce localized abscesses in various parts of the body. The invasion of the blood stream by double-zone hemolytic streptococci is not always fatal. We have on record a case of puerperal sepsis (retained placenta) in which the streptococci were found in the blood for nearly two months, followed by recovery. The prognosis for infections with these streptococci is more favorable than for infections with group A streptococci. The more than sixty strains in our collection from human autopsies and pathological conditions gives undue prominence to their pathogenicity. From 2000 autopsies cultured in this laboratory there were isolated 307 strains of beta-hemolytic streptococci and only 45 of these were double-zone streptococci. Very few of the latter could be suspected as the cause of death, and in many instances they were of no greater significance than other terminal or postmortem invaders. In other than a hospital population one might collect a large number of such strains without encountering pathological conditions. These streptococci are often harbored by normal individuals. They appear to be opportunist pathogens, as are many other organisms normally harbored by man, and there is no evidence that they cause contagious disease or are of epidemic significance.

There is evidence that the lactose +, salicin + and the lactose +, salicin − double-zone beta-hemolytic streptococci are of epizootic importance. Of the 45 strains from cow's milk, twenty-seven, including all but one of the salicin − strains, were from individual cows with clinical mastitis; five were received from other laboratories, labeled *Streptococcus mastitidis*; and the re-

maining fourteen were from mixed milk or were simply labeled "from milk" when received. Both the lactose +, salicin + and the lactose +, salicin − strains are causes of mastitis and probably are commonly transmitted from cow to cow during milking. R. B. Little (1937a) has shown that experimental mastitis may be produced with small amounts of culture introduced beyond the sphincter without injury to the teat, or by allowing the teat to aspirate culture or contaminated milk from a dish held in the hand (1937b). By the former method, Little (1938) produced mastitis in first-calf heifers with six double-zone beta-hemolytic streptococci isolated by us from human sources. See table 4.

In view of the above results there must be entertained the possibility of the infection of cows with double-zone streptococci

TABLE 4

Strains used for inoculation of cows udders

STRAIN	SOURCE	SEROLOGICAL		FERMENTATION	
		Group	Type	Lactose	Salicin
Nr.....	Septicemia following abortion	B	III	+	+
Fy.....	Normal throat	B	II	+	+
T78.....	Removed tonsil	B	Unclassified	+	+
BB.....	Blood of new-born infant	B	Ib	−	+
Gn.....	Normal throat	B	Ib	−	+
Py.....	Urine: cystitis	B	III	−	+

of human origin by the hands of milkers. That it may not happen frequently is indicated by the relative infrequency of the infection of the udder by group A hemolytic streptococci and by the absence, to date, of reports of the isolation of lactose −, group B hemolytic streptococci from the udder. It would appear that the human carrier needs to be considered, in attempts to eradicate mastitis from herds of cattle.

Among the double-zone beta-hemolytic streptococci studied were five from other animals than cows (table 3). Three strains were from guinea pigs, two from infected eyes and one from a foot, reported by Parsons and Hyde (1928). One strain was from a rabbit (Lancefield's strain K 158 A) used for testing vaccine virus. The above four strains were lactose −, salicin +.

The fifth strain was lactose +, salicin + and was isolated in 1908 from a horse with the diagnosis of "strangles." The stock strain has not been subjected to animal passage since its isolation. No other details of the case are known.

The lactose + double-zone beta-hemolytic streptococci would fall into "Group Ib" mastitis streptococci as described by Minett (1935), the *Streptococcus agalactiae* of some authors and the *Streptococcus mastitidis* of others. The tendency of some is to recognize both hemolytic and non-hemolytic varieties under these species, since both are common causes of mastitis and some of the non-hemolytic strains are reported to belong to serological group B and to be similar to the hemolytic strains except with regard to hemolysis. However, only a limited number of cultural tests have been employed and the discovery of other tests may reveal significant differences as was the case with *Streptococcus pyogenes* and some of the animal strains of serological group C previously indistinguishable from *Streptococcus pyogenes*. Frost, Gumm and Thomas (1927) proposed the name *Streptococcus asalignus* for the salicin − hemolytic streptococcus from milk which probably is identical with our lactose +, salicin − double-zone hemolytic streptococcus. Frost and Engelbrecht (in press) propose the name *Streptococcus mastitidis* for the hemolytic lactose +, salicin + mastitis streptococcus and the name *Streptococcus agalactiae* for the otherwise similar non-hemolytic mastitis streptococcus. There is equally good reason for giving a specific name to the lactose −, salicin + double-zone beta-hemolytic streptococcus belonging to serological group B. It would be a convenience to be able to designate these cultural entities by specific names, since the use of more general terms, the letters of the alphabet and even the Roman and Arabic numerals is becoming confusing and is meaningless without reference to the individual author cited. However, this is a situation which probably should be tolerated until more is known about the meaning of bacterial species in a rapidly developing field of study. The inclusion of more than one species in a single serological group need be no deterrent, since most authors recognize several species of hemolytic streptococci in serological groups A, C and

D. Neither need the finding of similar type antigens in bacteria of different species or genera be disturbing (recently discussed by Lancefield, 1938). It would appear that a species of *Streptococcus* need not be limited to a single serological type but should not transcend a serological group. I question whether streptococci of the same serological group and type need be placed in the same species if they show stable cultural differences.

No instability nor variability has been noted in the strains of double-zone beta-hemolytic streptococci in our possession although some of them have been under observation for twenty-five years.

SUMMARY AND CONCLUSIONS

Double-zone beta-hemolytic streptococci are found in the throats and vaginae of many normal persons. They are also a common cause of mastitis in cows.

These streptococci fall into three fermentative groups: lactose −, salicin +; lactose +, salicin +; and lactose +, salicin −.

All of the 188 strains examined belong to Lancefield's serological group B by the precipitin test.

In man, the lactose −, salicin + and the lactose +, salicin + strains may assume the rôle of opportunist pathogens, producing infections in various parts of the body and rarely may cause fatal septicemia. The source of such infections may usually be traced to the throat or genito-urinary tract. There is no evidence that these streptococci are of epidemic significance.

In cows, the lactose +, salicin + and the lactose +, salicin − strains are common causes of mastitis. The lactose −, salicin + strains have not been found in cows although they are capable of producing mastitis when inoculated into the udder.

In attempts to eradicate mastitis from dairy herds the human carrier of these streptococci should be considered.

The recognition of the double-zone hemolytic streptoocci supplies additional reason why diagnostic laboratories should no longer report all hemolytic streptococci as *Streptococcus hemolyticus*, a term which is inadequately informative and of doubtful validity as a specific name.

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REFERENCES

BROWN, J. H. 1937a Significance of the hemolytic streptococci found in milk. Cornell Veterinarian, **27**: 110–121.

BROWN, J. H. 1937b Appearance of double-zone beta hemolytic streptococci in blood agar. Jour. Bact., **34**: 35–48.

BROWN, J. H. 1938 A simplified method for grouping hemolytic streptococci by the precipitin reaction. Jour. Am. Med. Assoc., **111**: 310–311.

FROST, W. D., GUMM, M. AND THOMAS, R. C. 1927 Types of hemolytic streptococci in certified milk. Jour. Inf. Dis., **40**: 698–705.

HARE, R. AND MAXTED, W. R. 1935 The classification of hemolytic streptococci from the stools of normal pregnant women and of cases of scarlet fever by means of precipitin and biochemical tests. Jour. Path. and Bact., **41**: 513–520.

LANCEFIELD, R. C. 1933 A serological differentiation of human and other groups of hemolytic streptococci. Jour. Exp. Med., **57**: 571–595.

LANCEFIELD, R. C. 1938 Two serological types of group B hemolytic streptococci with related, but not identical, type-specific substances. Jour. Exp. Med., **67**: 25–40.

LITTLE, R. B. 1937a Bovine mastitis. I. The significance of the dose factor in the production of experimental mastitis. Cornell Veterinarian, **27**: 297–308.

LITTLE, R. B. 1937b Bovine mastitis. II. The production of mastitis by the suction of streptococci into the duct of the teat. Cornell Veterinarian, **27**: 309–316.

LITTLE, R. B. 1938 The Significance of Human Double-zone Beta-Hemolytic Streptococci in the Udders of the Cow. Jour. Exp. Med., **68**: 905–911.

MINETT, F. C. 1935 Streptococcus mastitis in cattle; Bacteriology and preventive medicine. Proc. 12th Int. Vet. Congress, 511–532.

PARSONS, E. I. AND HYDE, R. R. 1928 Spontaneous streptococcus infections in guinea pigs. Am. Jour. Hyg., **8**: 356–385.

PLUMMER, H. 1934 The fermentation of sorbitol and trehalose by hemolytic streptococci from various sources. Jour. Bact., **27**: 465–472.

SMITH, F. R. and SHERMAN, J. M. 1938 The hemolytic streptococci of human feces. Jour. Inf. Dis. **62**: 186–189.

TILLETT, W. S. AND GARNER, R. L. 1933 The fibrinolytic activity of hemolytic streptococci. Jour. Exp. Med., **58**: 485–502.