THE CULTURAL DIFFERENTIATION OF BETA HEMOLYTIC STREPTOCOCCI OF HUMAN AND BOVINE ORIGIN.

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Hemolytic streptococci are common in good dairy products and are usually harmless to the consumer. It is desirable to be able to distinguish such streptococci from hemolytic streptococci pathogenic to man which are at times found in dairy products. Up to the present there has been described no qualitative method for distinguishing hemolytic streptococci of human and bovine origin. Our ability to differentiate them depends upon the recognition of certain quantitative differences such as the agglutination or precipitation titer against an immune serum, the rapidity with which hemolysis appears, the size or definiteness of the hemolyzed zone in a blood agar plate, the rate of coagulation of milk, the degree of acidity produced in carbohydrate media, and the action of a bouillon culture on blood corpuscles in suspension. Anything which tends to make the determination of these quantitative differences more evident and simple is of value. Acquaintance with the minute details of appearances in blood agar is useful (1). Avery and Cullen (2) have shown the advantage of the determination of hydrogen ion concentration in dextrose bouillon cultures. We wish to emphasize the differential value of the action of streptococci of human and bovine origin on blood corpuscles in fluid media. The literature on the subject has been reviewed in a previous article.[1]

Action of Hemolytic Streptococci on Blood in Fluid Media.

The Medical Department of the United States Army in 1918 recommended the following method for the identification of *Streptococcus hæmolyticus* (3).

[1] Brown (1), Table IV.

"3. 0.5 cc. of the bouillon culture should be mixed with 0.5 cc. of a 5% suspension of washed rabbit blood corpuscles in physiological salt solution and incubated in a water bath at 37°C. for 2 hours. Markedly hemolytic pathogenic streptococci of human origin produce laking of blood under these conditions."

In Table I is recorded the result of the application of the above test to twenty-eight strains of streptococci of bovine and human origin. The strains tabulated were selected as representative of a large number of hemolytic bovine and human streptococci encountered by the author.

Since 1915 I have employed a slightly different technique from that described above for the same purpose and with practically the same results. A fresh young (18 to 20 hours old) standard veal or beef infusion bouillon culture is diluted to twenty times its volume with sterile 0.85 per cent salt solution. To 1 cc. of the diluted culture in small tubes in a Wassermann bath is added one drop of sterile defibrinated blood. The Wassermann tubes should be clean but need not be sterile. 2 hours incubation does not give opportunity for the growth of contaminating organisms to interfere with the reaction. The result of the application of this technique to the same streptococci as employed above is also shown in Table I.

The technique is perhaps a little simpler than that employed by the Army. It must not be assumed, however, that all mixtures of blood or blood corpuscles and bouillon culture will give similar results. It is a purely quantitative test and if it is to be of the maximum differential value the proportions described above must be adhered to. If the culture is diluted 1:10 some of the bovine strains produce hemolysis within the 2 hour period. If it is diluted 1:40 some of the human strains fail to produce hemolysis. Bouillon cannot be used instead of salt solution for making the dilutions. If more than one drop of blood is added the results are irregular. The bouillon cultures must be young and active. Experiments with centrifuged organisms indicate that the number of organisms present is of relatively little importance. The desired result attained by dilution of the culture appears to be due to dilution of the medium rather than dilution of the bacterial suspension. It is necessary to use a greater dilution of the culture when defibrinated blood is employed rather than washed corpuscles because the serum of the former serves as

TABLE I.

Action of Streptococci on Blood in Salt Solution and Bouillon.

Strain.	Source.	Hemolysis in 2 hrs. at 37°C.				
		Washed rabbit corpuscles. U. S. Army technique.	Defibrinated blood. Author's technique.			
			Horse.	Human.	Rabbit.	Beef.
A-Cow 1	Milk from individual cow.	−	−	−		
A-Cow 8	" " " "	−	−	−		
A-Cow 18	" " " "	−	−	−	−	−
A-Cow 25	" " " "	−	−			
B-Cow 18	" " " "	−	−	−	−	−
H-Cow 71	" " " "	−	−	−	−	−
H-Cow 72	" " " "	+	−	−	−	−
J-C10	" " " "	−	−	−		
J-C59	" " " " (mastitis).	−	−	−		
J-MJ	Milk pail.	−	−	−		
J-E7	" from individual cow.	−	−	−		
J-C65	Udder of cow at autopsy (mastitis).	−	+	±		
Cheese 1	Cream cheese.	−	−	−	−	−
" 2	" "	−	−	−	±	−
B-Cow 2b	Milk from individual cow (mastitis).	++++	+++	++		
H-Cow 108	Milk from individual cow (mastitis).	++++	++++	+++	++++	+++
H-M	Human throat.	+++	+++			
X-32	" "	++++	+++	++	+++	+++
X-38	" lymph node.	++++	+++	++	+++	±
X-40	" spleen.	++++	++++	+++	++++	+++
X-43	" kidney.	+++	++++	++++		
Imp. 1	Impetego contagiosa.	+++	++++	±		
" 2	" "	++++	++++	+++		
D-AD4	Human throat.	++++	++++	++++		
X-44	" empyema.	++++	++++	++		
X-45	" "	++++	++++	++++	++++	++++
X-46	" "	++++	++++	++++	++++	++++
A-SH	" peritoneal cavity.	++++	++++	++++		
Control.	Sterile salt solution.	−	−	−	−	−

Complete hemolysis is indicated by++++. Tubes were shaken each half hour.

medium for the streptococci. The importance of proper dilution and the influence of the length of period of incubation are shown in Table II. This table also illustrates strikingly the difference in hemolytic activity of bovine and human strains under these conditions.

TABLE II.

Action of Diluted Cultures on Defibrinated Blood. Effect of Dilution and Length of Incubation.

Strain and dilution.	Incubation at 37°C.				In room over night.	Strain and dilution.	Incubation at 37°C.				In room over night.
	½ hr.	1 hr.	1½ hrs.	2 hrs.			½ hr.	1 hr.	1½ hrs.	2 hrs.	
A-Cow 1						B-Cow 2b					
1: 5	−	−	−	±	+	1: 5	−	±	++++	++++	++++
1: 10	−	−	−	−	+	1: 10	−	±	+++	+++	++++
1: 20	−	−	−	−	+	1: 20	−	−	±	++	++++
1: 40	−	−	−	−	−	1: 40	−	−	−	+	++
1: 80	−	−	−	−	−	1: 80	−	−	−	−	+
A-Cow 8						H-Cow 108					
1: 5	−	−	−	±	+	1: 5	+++	++++	++++	++++	++++
1: 10	−	−	−	−	+	1: 10	+++	++++	++++	++++	++++
1: 20	−	−	−	−	+	1: 20	+++	++++	++++	++++	++++
1: 40	−	−	−	−	−	1: 40	++	+++	++++	++++	++++
1: 80	−	−	−	−	−	1: 80	−	++	+++	++++	++++
A-Cow 18						H-M					
1: 5	−	−	−	±	+	1: 5	+++	++++	++++	++++	++++
1: 10	−	−	−	−	+	1: 10	±	++	++++	++++	++++
1: 20	−	−	−	−	±	1: 20	−	±	+++	+++	++++
1: 40	−	−	−	−	−	1: 40	−	−	−	++	++++
1: 80	−	−	−	−	−	1: 80	−	−	−	+	+
A-Cow 25						X-32					
1: 5	−	−	−	±	++	1: 5	++	++++	++++	++++	++++
1: 10	−	−	−	−	+	1: 10	±	++	++++	++++	++++
1: 20	−	−	−	−	+	1: 20	−	±	+++	+++	++++
1: 40	−	−	−	−	−	1: 40	−	−	±	+	++++
1: 80	−	−	−	−	−	1: 80	−	−	−	±	++

B-Cow 18						X-38					
1:5	−	−	−	−	+	1:5	+++	++++	++++	++++	++++
1:10	−	−	−	−	+	1:10	±	+++	++++	++++	++++
1:20	−	−	−	−	+	1:20	−	+	++++	++++	++++
1:40	−	−	−	−	−	1:40	−	±	++	+++	++++
1:80	−	−	−	−	−	1:80	−	−	−	++	++++
H-Cow 71						X-40					
1:5	−	−	−	−	−	1:5	++++	++++	++++	++++	++++
1:10	−	−	−	−	−	1:10	+++	++++	++++	++++	++++
1:20	−	−	−	−	−	1:20	+	++++	++++	++++	++++
1:40	−	−	−	−	−	1:40	−	+++	++++	++++	++++
1:80	−	−	−	−	−	1:80	−	±	++++	++++	++++
H-Cow 72						X-43					
1:5	−	−	+	++	++++	1:5	++	++++	++++	++++	++++
1:10	−	−	−	+	+++	1:10	±	+++	++++	++++	++++
1:20	−	−	−	−	++	1:20	−	++	++++	++++	++++
1:40	−	−	−	−	+	1:40	−	+	++++	++++	++++
1:80	−	−	−	−	+	1:80	−	−	++	++++	++++
J-C10						Imp. 1					
1:5	−	−	−	−	−	1:5	++	++++	++++	++++	++++
1:10	−	−	−	−	−	1:10	±	+++	++++	++++	++++
1:20	−	−	−	−	−	1:20	−	+	++++	++++	++++
1:40	−	−	−	−	−	1:40	−	−	++++	++++	++++
1:80	−	−	−	−	−	1:80	−	−	−	++++	++++

The tubes were shaken after each reading to keep the blood corpuscles from settling out.

TABLE II—*Concluded.*

Strain and dilution.	Incubation at 37°C.				In room over night.	Strain and dilution.	Incubation at 37°C.				In room over night.
	½ hr.	1 hr.	1½ hrs.	2 hrs.			½ hr.	1 hr.	1½ hrs.	2 hrs.	
J-C59						Imp. 2					
1: 5	−	−	−	±	++	1: 5	+++	++++	++++	++++	++++
1: 10	−	−	−	−	++	1: 10	++	++++	++++	++++	++++
1: 20	−	−	−	−	+	1: 20	−	+++	++++	++++	++++
1: 40	−	−	−	−	+	1: 40	−	±	++++	++++	++++
1: 80	−	−	−	−	+	1: 80	−	−	++	++++	++++
J-MJ						D-AD4					
1: 5	−	−	±	+++	++++	1: 5	++	++++	++++	++++	++++
1: 10	−	−	−	−	+++	1: 10	++	++++	++++	++++	++++
1: 20	−	−	−	−	+	1: 20	+	++++	++++	++++	++++
1: 40	−	−	−	−	−	1: 40	−	+++	++++	++++	++++
1: 80	−	−	−	−	−	1: 80	−	−	++++	++++	++++
J-E7						X-44					
1: 5	−	−	−	−	−	1: 5	+++	++++	++++	++++	++++
1: 10	−	−	−	−	−	1: 10	±	+++	++++	++++	++++
1: 20	−	−	−	−	−	1: 20	−	+	++++	++++	++++
1: 40	−	−	−	−	−	1: 40	−	−	+++	++++	++++
1: 80	−	−	−	−	−	1: 80	−	−	−	+	++
J-C65						X-45					
1: 5	−	−	−	±	++	1: 5	++++	++++	++++	++++	++++
1: 10	−	−	−	−	+	1: 10	++++	++++	++++	++++	++++
1: 20	−	−	−	−	+	1: 20	+++	++++	++++	++++	++++
1: 40	−	−	−	−	+	1: 40	++	++++	++++	++++	++++
1: 80	−	−	−	−	+	1: 80	±	+++	++++	++++	++++

Cheese 1										
1:5	−	−	−	±	+	X-46				
1:10	−	−	−	−	+	1:5	++++	++++	++++	++++
1:20	−	−	−	−	±	1:10	++++	++++	++++	++++
1:40	−	−	−	−	−	1:20	+++	++++	++++	++++
1:80	−	−	−	−	−	1:40	++	+++	++++	++++
						1:80	±	++	++++	++++
Cheese 2						A-SH				
1:5	−	−	−	±	+++	1:5	++++	++++	++++	++++
1:10	−	−	−	−	+	1:10	++	++++	++++	++++
1:20	−	−	−	−	+	1:20	+	+++	++++	++++
1:40	−	−	−	−	+	1:40	−	++	++++	++++
1:80	−	−	−	−	−	1:80	−	−	++	++++

Appearance in the Blood Agar Plate.

In the blood agar plate all the strains selected belong to the beta type. They show certain quantitative differences, however, as indicated in Table III.

TABLE III.

Hemolysis in Horse Blood Agar.

Strain.	Character of the zone produced by the deep colony.
A-Cow 1 A-Cow 8 A-Cow 18* A-Cow 25	A small clear sharply defined central zone and a broad outer partly hemolyzed zone.
B-Cow 18* H-Cow 71 H-Cow 72 J-C10 J-C59 J-MJ	The zone is smaller and slower to develop than that of known human strains.
J-E7 J-C65 Cheese 1 " 2	The zone is a little slower to develop than that of human strains but might be taken for the latter.
B-Cow 2b* H-Cow 108 H-M X-32 X-38* X-40 X-43 Imp. 1 " 2 D-AD4* X-44 X-45 X-46 A-SH	Typical human strains. Clear colorless well defined completely hemolyzed zone 2–2.5 mm. in diameter after incubation for 18–24 hrs. at 37°C.

* Blood agar plates of these strains are illustrated in a previous article (1).

The zones of hemolysis produced by the pathogenic beta type hemolytic streptococci of human origin are of uniform size and character, but those produced by the bovine strains show considerable variety. It is, therefore, often possible on seeing a deep colony in

a blood agar plate for the first time to state with a fair degree of certainty that the organism is not of the human pathogenic variety. However, since some bovine streptococci produce zones much like those of human strains, it is not possible to state positively that such a colony is of human origin. Four such strains are indicated in Table III.

Fermentation Reactions.

In Table IV is given the titratable acidity after incubation for 1 week of cultures in fermented bouillon plus 5 per cent of sterile horse serum and 1 per cent of the test substance indicated.

TABLE IV.

Fermentation Reactions.

Strain.	Titratable acid (per cent normal).					
	Saccharose.	Lactose.	Salicin.	Raffinose.	Inulin.	Mannite.
A-Cow 1	4.65	4.4	1.4	1.15	1.15	0.95
A-Cow 8	4.8	4.85	1.45	1.1	1.15	1.0
A-Cow 18	4.7	5.0	1.48	1.3	1.0	1.0
A-Cow 25	4.75	5.0	1.15	1.1	1.1	1.1
B-Cow 18	3.85	5.0	1.2	0.95	0.95	0.9
H-Cow 71	4.9	5.0	3.3	0.55	0.55	0.65
H-Cow 72	4.95	4.85	3.25	0.55	0.7	0.5
J-C10	5.3	4.8	4.4	0.6	0.55	0.3
J-C59	4.95	4.7	3.8	0.65	0.6	0.45
J-MJ	3.45	3.35	0.5	0.45	0.5	0.55
J-E7	4.75	4.6	3.7	0.5	0.55	0.5
J-C65	4.45	3.15	3.25	0.7	0.2	0.3
Cheese 1	5.7	4.7	5.0	0.8	0.35	3.8
" 2	3.4	4.45	5.6	0.8	0.3	3.65
B-Cow 2b	3.6	3.65	4.8	1.05	0.9	0.85
H-Cow 108	3.6	3.2	3.2	1.0	1.0	0.8
H-M	3.55	3.7	3.55	0.8	0.6	0.85
X-32	3.55	3.55	4.7	0.9	0.75	0.95
X-38	3.95	3.65	4.8	0.8	0.85	0.8
X-40	3.85	3.15	4.9	0.8	0.8	0.7
X-43	3.9	3.25	3.4	1.05	1.0	1.0
Imp. 1	4.0	3.4	3.55	0.55	0.9	0.7
" 2	2.9	3.5	2.95	0.6	0.5	0.35
D-AD4	3.25	2.85	2.65	0.85	0.85	0.7
X-44	3.7	3.45	3.35	0.4	0.75	0.7
X-45	3.9	2.8	2.5	0.35	0.25	0.3
X-46	3.6	3.75	3.8	0.35	0.1	1.5
A-SH	3.65	1.0	4.35	1.0	1.2	3.4

The titratable acidity of the medium was 0.5 to 1 per cent normal. A titratable acidity of less than 1.5 is regarded as a negative fermentation reaction.

Limiting Hydrogen Ion Concentration.

In Table V is given the hydrogen ion concentration of cultures in 1 per cent dextrose bouillon after incubation for 68 hours.

TABLE V.

Limiting Hydrogen Ion Concentration in Dextrose Bouillon.

Strain.	pH				Strain.	pH
	24 hrs.	68 hrs.	72 hrs.	116 hrs.		68 hrs.
A-Cow 1		4.6			B-Cow 2b	5.2
A-Cow 8		4.5			H-Cow 108	5.2
A-Cow 18		4.6			H-M	5.2
A-Cow 25		4.5			X-32	5.2
B-Cow 18		4.4			X-38	5.2
H-Cow 71		4.5			X-40	5.1
H-Cow 72		4.6			X-43	5.2
J-C10		4.6			Imp. 1	5.3
J-C59		4.5			" 2	5.3
J-MJ	5.8	5.3		5.3	D-AD4	5.3
J-E7	5.1	4.6	4.3		X-44	5.4
J-C65		5.1			X-45	5.2
Cheese 1		4.4			X-46	5.2
" 2		4.4			A-SH	5.2

Hydrogen ion determinations were made colorimetrically according to Clark and Lubs (Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191) with methyl red as an indicator.

The streptococci studied by Avery and Cullen (2) reached their limiting hydrogen ion concentration within 24 hours. This was also true of all the known human strains and most of the bovine strains studied by the author. However, a few of the latter did not; *e.g.*, Strain J-E7. Neither could Strain J-MJ be depended upon in this respect. It was also noted that some substances, *e.g.* salicin, were fermented more slowly than others. The limiting hydrogen ion concentration was reached later in media containing this substance.

Growth in Milk.

Reference to Table VI shows that no sharp distinction between bovine and human strains can be drawn from their ability to coagulate milk in test-tubes. After incubation for 5 or 6 days most of the

strains, human and bovine, had coagulated the milk. In 24 hours only about half the bovine strains had done so. In 48 hours some of the human strains had partially coagulated the milk and some of the bovine strains had done no better. The most that can be said is that none of the strains of human origin caused coagulation of milk within 24 hours, whereas about 50 per cent of the bovine strains did so.

TABLE VI.

Coagulation of Milk.

Strain.	Period of incubation.			Strain.	Period of incubation.		
	24 hrs.	48 hrs.	6 days.		24 hrs.	48 hrs.	6 days.
A-Cow 1	++	++++	++++	B-Cow 2b	−	−	++++
A-Cow 8	++	++++	++++	H-Cow 108	−	++	++++
A-Cow 18	+++	++++	++++	H-M	−	++	++++
A-Cow 25	+++	++++	++++	X-32	−	−	+++
B-Cow 18	+++	++++	++++	X-38	−	+	+++
H-Cow 71	++	++++	++++	X-40	−	−	±*
H-Cow 72	+++	++++	++++	X-43	−	−	+++
J-C10	−	+	++++	Imp. 1	−	+	+++
J-C59	−	+	++++	" 2	−	−	−*
J-MJ	−	−	−*	D-AD4	−	−	−*
J-E7	+	++	++++	X-44	−	++	++++
J-C65	−	−	−*	X-45	−	+	++++
Cheese 1	−	+	++++	X-46	−	+	++++
" 2	++++	++++	++++	A-SH	−	−	−†

* Coagulated when placed in boiling water.

† Not coagulated when placed in boiling water.

++++ indicates complete coagulation.

Reaction to Methylene Blue.

It has been claimed by Sherman and Albus (4) that cultures of *Streptococcus lacticus* reduce methylene blue in milk while those of *Streptococcus pyogenes* fail to do so and in fact fail to grow in this medium. Their results are striking, though we doubt the validity of their method of selecting cultures. We cannot agree in applying the name *Streptococcus pyogenes* to all streptococci isolated directly from the udder. The strains studied above were inoculated into methylene blue milk (1: 20,000) according to the technique described by Sherman

and Albus. The two strains from cheese reduced the methylene blue almost completely in less than 16 hours, but certain strains from the udder and from human patients also produced partial reduction in less than 24 hours, others within 66 hours, and others not at all. On methylene blue agar plates (1: 20,000) streaks of Strains Cheese 1 and Cheese 2 grew luxuriantly, while none of the other strains grew at all. It is interesting to note, however, that streptococci which were inhibited by methylene blue in the presence of oxygen grew well in media containing methylene blue which was reduced by some other agent. This was demonstrated by boiling tubes of methylene blue agar in a water bath until the dye was colorless, inoculating the agar in fluid condition, chilling it quickly, and incubating. After several hours the blue color returned to the upper half inch of the medium and in this portion no colonies developed. Below the upper half inch the agar remained colorless and here the streptococcus colonies grew as well as in agar containing no methylene blue.

CONCLUSION.

None of the procedures described serves by itself to differentiate streptococci of human and bovine origin with certainty, though each of them serves as a strong presumptive test. Most strains fall easily into the human or bovine group by all the tests. Eliminating these from consideration we have left certain irregular strains listed in Table VII.

TABLE VII.

Irregular Strains.

Strain.	Source.	Origin as indicated by.		
		Blood agar plate.	Blood-salt solution.	Acidity n dextrose bouillon.
J-E7	Normal udder.	Human (doubtful).	Bovine.	Bovine.
Cheese 1	Cream cheese.	" "	"	"
" 2	" "	" "	"	"
J-C65	Diseased udder.	" "	"	Human.
J-MJ	Milk pail.	Bovine.	"	"

Taking all characters into consideration we are inclined to regard Strains J-E7, Cheese 1, and Cheese 2 as undoubtedly of bovine origin. Strain J-MJ also is representative of a group of streptococci which Jones[2] has found in milk and which is being further studied by him. There remains Strain J-C65 which for the present must be regarded as of doubtful origin.

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[2] Jones, F. S., personal communication.