

## A STUDY OF THE STREPTOCOCCI FROM FIFTY CASES OF BOVINE MASTITIS

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### INTRODUCTION

It is claimed by most recent workers on bovine mastitis that the majority of streptococcus strains which can be isolated from this condition belong to a fairly well-defined group to which the specific name *Streptococcus mastitidis contagiosae* is sometimes applied or alternatively *Streptococcus agalactiae*.

According to Klimmer, Haupt and Roots (1928) and Haupt (1931) it was only in isolated instances that mastitis is caused by streptococci other than *Str. agalactiae*. Diernhofer (1930), on the other hand, expressed the opinion that cases due to that type of streptococcus are comparatively rare. Supporting the latter view, Reis and Sevansson (1931) apparently did not encounter the so-called *Str. mastitidis* in typical cases of mastitis and could incriminate no particular species from a heterogeneous streptococcal flora. Minett (1932) stated that the streptococci which cause the chronic form of mastitis constitute a clearly defined group for which the name *Str. agalactiae* has come into general use in Central Europe. This organism presented similar characters to that originally isolated in 1887 by Nocard and Mollereau (1887) from a case of "contagious bovine mammitis". As a rule, haemolytic properties were not very marked, although the strains varied in this respect. Certain strains exhibited  $\beta$  haemolysis as described by Brown (1919) around deep colonies on blood agar plates, although the zones were narrow and the margins usually indistinct. Other strains gave haemolysis of the type  $\alpha$  or  $\alpha'$ . Recent work published by S. J. Edwards (1932) demonstrated that many of these streptococci from mastitis could be included in a single group possessing cultural and biochemical characters distinguishing them from *Str. agalactiae*. There is reason to believe that hitherto these streptococci were confused with *Str. lactis*, even though they presented many different properties. A third type of streptococcus was isolated by Minett and his co-workers (1930) from bovine mastitis and differed from *Str. agalactiae*, amongst other respects, in that it was strongly haemolytic and highly virulent for the small laboratory animals. This type did not differ in any important point from *Str. pyogenes* of man. On first isolation these strains frequently possessed definite capsules, and thus the colonies grown on blood agar in a moist atmosphere were mucoid and striated. Since similar organisms were reported by Frost (1932), Savage (1906-7) and

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Armstrong and Parran (1927) from milk-borne epidemics of septic angina, the term *Str. epidemicus* has been applied to them, especially in the United States.

Seelemann (1932), as a result of an extended study of bovine mastitis, stated that he had not found a single strain of streptococcus with the typical characteristics of *Str. mastitidis* which was also haemolytic. Similar statements have been made by Rudolf (1926), Kollenz (1924) and others, amongst whom Rosell (1933) reported that, of 400 strains isolated from mastitis in Canada and the United States, only twelve were  $\beta$ -haemolytic.

It will be seen from the preceding brief outline of the attempts to classify the streptococci of mastitis, that the problem in the bacteriology of the disease has been the difficulty of establishing a recognised species of streptococcus as the causative organism. So-called " $\beta$ -haemolytic" strains, bearing close resemblance to *Str. pyogenes* of human disease, as well as the "viridans" type, have frequently been isolated from mastitis secretion, but the methods of isolation and criteria of identification have been so varied and have deviated so much from the standards employed by workers in the study of streptococci of human origin, that it is difficult to recognise a common basis of classification.

The purpose of the present work, therefore, was to apply the methods of medical bacteriology to strains of streptococci from fifty cases of bovine mastitis. For comparison, the characteristics by which *Str. agalactiae* is defined by veterinary bacteriologists were also studied. In this way it was hoped to determine: (1) how bovine mastitis strains were related to the streptococci of man; (2) whether strains from a single clinical condition (bovine mastitis) were uniform in their biological characters. Such an endeavour was intended to throw some light on the difficult problems of classification within this bacterial group.

## EXPERIMENTAL WORK

### METHODS

#### *Source of material*

Samples were obtained from the udders of dairy cattle just after slaughter at the abattoir. The secretion was always purulent and in a few cases tinged with blood. Only material showing gross alteration in physical appearance was investigated. In every case the sample was obtained within an hour of slaughter, before the udder had become cold. The teat orifice was cleansed with hot izal solution and methylated spirit, the secretion from all quarters being collected in a sterile bottle. Samples were taken to the laboratory without delay and the examination was commenced at once.

#### *Technique of isolation*

Primary isolation was effected by means of poured blood agar plates, single colonies being subcultured on blood agar and boiled blood agar plates in series

to observe the action of the organism on the blood media and at the same time to ensure purity of the final culture. The details are as follows:

(1) The presence of streptococci and their relative number were estimated by the examination of Gram-stained films of secretion. In addition the cellular content of the material was studied in films stained by the Breed (1921) technique by which staining with methylene blue was combined with fat extraction.

(2) Cultures were made in the form of poured ox-blood agar plates inoculated each with a loopful of secretion, inoculum and melted agar being thoroughly mixed before the medium was allowed to set. After the surface had dried in the air oven at 45° C. the plates were incubated. When the initial film examination revealed that the streptococci were scanty the milk was centrifuged and the sediment used as inoculum in the same way.

(3) After incubation at 37° C. for 24–48 hours the plates were closely examined to determine the cultural type of streptococcus present. Twelve colonies were subcultured on the surface of an ox-blood agar plate suitably divided up by means of lines drawn on the glass with the grease pencil.

(4) After incubation a single colony was transferred from each section to a corresponding division of a boiled blood agar plate. This heated blood medium served for the identification of the viridans or  $\alpha$  type of colony.

(5) Examination of colony appearances on these two blood media revealed the predominant type of streptococcus present, and a single colony from the boiled blood agar plate was subinoculated into phosphate broth for further study. The repeated plating of isolated colonies was considered sufficient justification for accepting this final broth culture as being pure.

In only one instance was more than one cultural type of streptococcus detected in a single sample of udder secretion. In this case  $\beta$  haemolytic and  $\gamma$  types were associated. Both were studied. This fact accounts for fifty-one strains from the fifty cases of the series.

#### *Tests*

Each of the media used in the following tests was inoculated from a 24 hours' phosphate broth culture.

*Capsulation.* A tube of 5 per cent. ox-blood phosphate broth was incubated at 37° C. for 24 hours and a film stained by Hiss's method for capsule demonstration.

*Action on blood.* The changes produced by growth on 5 per cent. ox-blood agar after incubation for 48 hours at 37° C. was noted under low- and high-power magnification of the microscope according to the method described by Brown (1919), who classified the streptococci as of  $\alpha$ ,  $\alpha'$ ,  $\beta$  and  $\gamma$  types.

*Bile resistance.* This character was tested by inoculating a MacConkey plate and incubating at 37° C. for 5 days. The streptococcus was adjudged bile resistant if good growth occurred within that period.

*Growth in phosphate broth.* A broth culture was examined for the type of growth—turbidity and deposit.

*Colony appearance.* Isolated colonies on 5 per cent. ox-blood agar were examined by the naked eye and by low-power magnification of the microscope. Size, colour and granularity of the colony were noted as well as the appearance of the blood in the surrounding zone.

*Heat resistance.* The heat resistance was estimated by exposing 10 c.c. of a 24 hours' phosphate broth culture to a temperature of 60° C. in a water-bath for 15 min. A 5 per cent. ox-blood agar plate was inoculated with a loopful of the culture before and after heating, and after incubation at 37° C. for 48 hours, growth was noted and the action on blood further observed.

*Haemolytic action.* The degree of haemolysin formation in fluid culture was measured by inoculating a tube of 10 per cent. serum phosphate bouillon and incubating for 18 hours at 37° C.; 0.01–1 c.c. of whole culture was added to tubes containing 1 c.c. of 5 per cent. washed red blood cells in saline. The degree of lysis in each tube was observed after incubation for 1 hour at 37° C. and confirmed after standing overnight in the ice-chest. Ox, horse, sheep and pig red cells were used and controls included contained appropriate amounts of sterile 10 per cent. serum phosphate bouillon and 1 c.c. of washed red cells in saline.

*Fermentation reactions.* Sugar fermentation was tested on serum-peptone-water agar slopes tinted with litmus and containing 1 per cent. of the sugars—lactose, salicin, inulin, raffinose and mannite. The inoculated tubes were incubated for 5 days at 37° C. and inspected every 24 hours for acid production. In a number of the tests Hiss's serum water medium was used as the basis.

*Aesculin fermentation.* The ability to ferment the glucoside aesculin in the presence of bile salts was tested by inoculating a tube of aesculin-taurocholate fluid medium and incubating for 5 days at 37° C. Fermentation was indicated by the blackening of the medium.

*Changes in litmus milk.* Two tubes of litmus milk were inoculated and incubated, one at 37° C. for 5 days and the other at room temperature (15° C.) for 20 days. Acid production and consistency of clot were noted after 24 hours and after the respective periods.

*End pH of glucose broth culture.* A tube of 1 per cent. glucose broth (pH 7.4) was inoculated and incubated at 37° C. for 5 days, when the end pH attained by the culture was measured by colorimetric methods.

*Methylene-blue reductase production.* A tube of milk containing methylene blue in a concentration of 1 in 20,000 was inoculated and incubated for 5 days at 37° C. The tube was inspected after 24 hours and then after 5 days.

*Hydrolysis of sodium hippurate.* A tube of broth containing 1 per cent. of sodium hippurate was inoculated and incubated for 5 days at 37° C. To 1 c.c. of the clear supernatant, 0.2–0.4 c.c. of 12 per cent. ferric chloride solution, containing 2.5 c.c. of concentrated HCl per litre, was added and the tube immediately shaken. A precipitate appeared momentarily in the absence of

benzoate. If clearing did not occur, benzoate was considered to have been formed, since the hippurate in the uninoculated medium was previously determined to be soluble in the quantity of acid ferric chloride solution present. The presence of benzoate denoted the hydrolysis of the hippurate in the medium.

As this method did not constitute a recognised chemical test for benzoate, a test advocated by Plimmer (1933) was also applied.

The culture was acidified with just sufficient dilute HCl to render it acid to litmus. An extract of the culture was made with petrol ether in which benzoic but not hippuric acid is soluble. The layer of petrol ether, which had extracted any benzoic acid present in the culture, was pipetted off into another tube and evaporated to dryness in a water-bath kept at boiling-point. Excess of dilute ammonia was added to form ammonium benzoate if benzoic acid were present. The ammonia excess was removed by boiling until the solution was neutral. A few drops of *neutral* ferric chloride solution were added and gave a buff precipitate of ferric benzoate in the presence of benzoic acid.

#### RESULTS

In the following description of the cultural characteristics of different strains, those obtained from Prof. Minett as typical mastitis strains are termed *Str. agalactiae* (Minett) to distinguish them from those we have isolated in Edinburgh.

All strains of streptococci were primarily grouped according to the type of haemolysis— $\alpha$ ,  $\alpha'$ ,  $\beta$ , and  $\gamma$  types. The description should be read in conjunction with Tables I, II and III.

##### $\alpha$ (*alpha*) type (see Table I)

Fourteen of the fifty-one Edinburgh mastitis strains gave the appearances of  $\alpha$ -lysis in unaltered ox-blood agar and a definite green coloration on the heated blood medium. The latter criterion was considered of greater importance, since its recognition was less susceptible to personal factors in the examination of growth. The strains were then regarded as *Str. viridans* and their further properties examined. For comparison, viridans strains from the human throat were examined in the same culture media, as well as viridans strains from human urine, bovine faeces, a strain from bovine saliva—*Str. viridans bovis* (Belenky), two strains of *Str. agalactiae* (Minett) and a viridans strain from normal milk.

*Morphology and colonial appearances.* No significant differences were noted among strains from the various sources in the size and shape of individual cocci and in the length of chains produced in phosphate bouillon. In and on solid blood media, both human and bovine strains yielded small discrete colonies.

*Bile resistance.* On MacConkey's medium, nine of the fourteen mastitis viridans strains grew within 5 days, all the human viridans throat strains

Table I. *Strains of Str. viridans (α type) from cases of bovine mastitis compared with similar strains from other sources*

Title of strains	No. of strain	Growth on heated blood agar	Growth on MacConkey* 15 min.	Heat resistance for 60° C.	Lysis produced by 0.5 c.c. of fluid culture	Fermentation reactions						Biochemical type (Hofman)	Effect on litmus milk†		End of gluc bro	Methylene-blue reductase	Hydrolysis of hippurate	
						A	B	C	D	E	F		15° C.	37° C.			Precipitate	Extract
Edinburgh Mastitis strains (bovine mastitis)	14	Viridans growth +	+++ (4) +++ (3) ++ (2) + (5)	— (13) + (1)	Complete (1) Partial (1) No lysis (12)	+	+	+	—	—	—	1) faecalis (6) 5) ignavus (3) NH III (2) NH I (1) nitris (1) equinus (1)	+	+	<4.5 4.5-5.5	— (13) + (1)	— (8) + (6)	— (10) + (4)
Human throat strains	4	Do.	— (4)	—	Partial (1) No lysis (3)	+	—	+	+	—	—	nitris (4)	—	+	<4.5 4.5-5.5	—	—	—
Human urine strains	2	Do.	+++ (2)	—	No lysis (2)	+	—	+	+	+	—	nitris (2)	+	(1) — (1)	<4.5	—	—	—
Bovine faecal strains	6	Do.	+++ (6)	—	No lysis (6)	+	—	+	+	+	—	nitris (6)	—	—	<4.5	+	—	—
<i>Str. viridans bovis</i> (Belenky) (bovine saliva)	1	Do.	+++ (1)	+	No lysis (1)	+	+	+	—	+	—	faecalis (1)	+	+	<4.5	+	—	—
<i>Str. agalactiae</i> (Minetti)	2	Do.	+++ (1) — (1)	—	Partial lysis (2)	+	—	+	—	—	—	nitris (2)	—	—	<4.5	—	+	(2) + (2)
Normal milk strain	1	Do.	— (1)	—	No lysis (1)	—	+	—	—	—	—	NH III (1)	+	+	<4.5	—	—	—

A = lactose; B = mannite; C = salicin; D = raffinose; E = inulin; F = aesculin bile salt.

\* + + + = growth in 24 hours; + + = growth in 2 days; + = growth in 5 days.

† + + = acid and clot; + = acid.

‡ NH = "Non-haemolytic".

These notes also apply to Tables II and III.

Table II. Strains of Str. haemolyticus ( $\alpha'$  and  $\beta$  types) from cases of bovine mastitis compared with similar strains from other sources

No. of strains	Title of strains	Growth on heated blood agar	Growth on MacConkey	Heat resistance 60° C. for 15 min.	Lysis produced by 0.5 c.c. of fluid culture	Fermentation reactions						Biochemical type (Holman)	Effect on litmus milk†		End pH in glucose broth	Hydrolysis of hippurate	
						A	B	C	D	E	F		15° C.	37° C.			
11	Edinburgh Mastitis strains (bovine mastitis)	Greyish white growth	+++	—	Partial lysis (11)	+	+	+	±	—	—	pyogenes (9) anginosus (1) haemolyticus I (1)	+	+	4.0-4.6 (11)	— (10) + (1)	— (8) + (3)
1	Normal milk strain	Colourless growth	—	—	Partial lysis (1)	+	+	+	—	—	—	infrequens	—	+	4.3 (1)	—	—
6	Edinburgh Mastitis strains (bovine mastitis)	Greyish white growth	+++	— (2) + (1) (3)	Complete lysis (6)	+	+	+	—	—	—	pyogenes (4) anginosus (1) infrequens (1)	+	+	<4.5 (6)	(4) (2)	(4) (2)
4	Human throat strains	Colourless growth	—	—	Complete lysis (6)	+	+	+	—	—	—	pyogenes	—	+	4.5-5.5 (4)	—	—
2	Str. agalactiae (Minné)	Greyish white growth	+	— (1) (1)	Complete lysis (2)	+	+	+	—	—	—	pyogenes (1) anginosus (1)	—	+	<4.5 (2)	+	(2)

Table III. Strains of non-haemolytic streptococcus ( $\gamma$  type) from cases of bovine mastitis compared with similar strains from other sources

No. of strains	Title of strains	Growth on heated agar	Growth on MacConkey	Heat resistance 60° C. for 15 min.	Lysis produced by 0.5 c.c. of fluid culture	Fermentation reactions						Biochemical type (Holman)	Effect on litmus milk†		End pH in glucose broth	Hydrolysis of hippurate	
						A	B	C	D	E	F		15° C.	37° C.			
—	Edinburgh Mastitis strains (bovine mastitis)	Greyish white growth	+++	— (10) + (4) (7)	No lysis	+	+	+	±	—	—	mitis (7) ignavus (5) faecalis (3) 1) NHI (3) 2) salivarius (2)	— (11) +	— (4) +	<4.5 (14) 4.5-5.5 (5) 5.6 (1)	— (12) — (8)	— (6) — (14)
—	Human faecal strains	Do.	+++	+	No lysis	+	+	+	—	—	+	faecalis	+	+	<4.5 (2)	—	—
—	Normal milk strains	Do.	+++ (1) (1)	— (1)	No lysis	+	+	+	—	—	—	faecalis (1) NHI (1)	+	+	<4.5 (2)	—	—
—	MA strain (Mastitis Anderson)	Do.	—	—	No lysis	+	+	+	—	—	—	mitis	—	+	<4.5 (1)	+	+
—	Str. agalactiae (Minné)	Do.	+++ (1) (1) (1)	—	No lysis	+	+	+	—	—	—	salivarius	—	+	<4.5 (2) 4.5-5.5 (1)	+	(3)

failed to grow, while one *Str. agalactiae* (Minett) strain grew well and the other failed to grow. Both human urinary strains, the six bovine faecal strains and *Str. viridans bovis* (Belenky) grew well on MacConkey's medium, but the normal milk strain failed to grow.

*Growth in phosphate bouillon.* Bovine and human strains alike produced a dense turbidity with a heavy granular deposit in 24 hours, with the exception of four of the six bovine faecal strains which produced a flocculent deposit with clear supernatant and three of the fourteen mastitis strains which produced a diffuse turbidity with no deposit.

*Heat resistance.* Only one of our fourteen mastitis strains of  $\alpha$  type resisted 60° C. for 15 min. as did *Str. viridans bovis* (Belenky) with which it was identical. All other strains failed to resist 60° C. for 15 min.

*Haemolytic action.* Two of the fourteen viridans strains of our mastitis series produced complete lysis of the test volume of red blood cells, one with 0.5 c.c. and the other with 1 c.c. of whole fluid culture. One of the four human viridans throat strains and both *Str. agalactiae* (Minett) strains produced complete lysis with 1.0 c.c. of whole fluid culture. No other viridans strain formed haemolysin in fluid culture.

*Biochemical reactions.* According to Holman's classification six of the fourteen mastitis strains belonged to the faecalis type, three to the ignavus type, two to the non-haemolyticus III type, one to the non-haemolyticus I type, one to the mitis type and one to the equinus type. The four human viridans throat strains belonged to the mitis type as did both strains of *Str. agalactiae* (Minett), the two human urinary strains and the six bovine faecal strains. *Str. viridans bovis* (Belenky) belonged to the faecalis type and the normal milk strain to the non-haemolyticus III type.

*Aesculin fermentation.* Only one of fourteen viridans strains of our series fermented this substance in a bile-salt medium. The two human urinary strains and the *Str. viridans bovis* (Belenky) strain also blackened it. All other strains failed to ferment the aesculin in the medium.

*Changes in litmus milk.* Only two of the fourteen Edinburgh mastitis strains acidified litmus milk at 15° C., and one strain formed acid and four acid and clot at 37° C. The four human viridans throat strains failed to acidify milk at 15° C. but formed acid and clot at 37° C. One human urinary strain acidified milk at 15° C. and the other failed to do so, but both acidified milk at 37° C. None of the bovine faecal strains formed acid in milk at 15° C., but three of the six strains acidified milk at 37° C. and the other three formed acid and clot. The *Str. viridans bovis* (Belenky) strain acidified milk at 15 and 37° C. The two *Str. agalactiae* (Minett) strains acidified milk at 37° C. but not at 15° C. The strain from normal milk formed acid in milk at 15° C. and acid and clot at 37° C.

*End pH of glucose bouillon culture.* Eleven of our fourteen mastitis strains of  $\alpha$  type produced an end pH of 4.5 or less and three an end pH of 4.5-5.6. Three of the four human viridans throat strains produced an end pH of 4.5

or less and one an end pH of 4.5-5.5. All other strains attained a final pH of 4.5 or less.

*Methylene-blue reductase production.* Only one of the fourteen viridans strains from Edinburgh cases of mastitis reduced methylene-blue milk. All four human throat strains failed to reduce the dye as did both strains of *Str. agalactiae* (Minett) and the normal milk strain. The two human urinary strains, five of the six bovine faecal strains and *Str. viridans bovis* (Belenky) all produced a reductase for methylene blue.

*Hydrolysis of sodium hippurate.* Four of the fourteen  $\alpha$ -haemolytic strains hydrolysed hippurate as did both strains of *Str. agalactiae* (Minett). All other strains failed to hydrolyse hippurate.

*Summary of  $\alpha$  strains.*

Of the fourteen mastitis strains which were of viridans type, one fermented raffinose but not mannite as does the common human throat viridans type but it differed from the latter in other respects. Three of our mastitis strains closely resembled *Str. viridans* of man except that in each case mannite was fermented and not raffinose. Among the other strains the following deviations from the usual characteristics of the human viridans type were noted:

Growth on MacConkey's medium ... ..	9 strains
Resistance to 60° C. for 15 min. ... ..	1 ,,
Haemolysin in fluid medium ... ..	1 ,,
Hydrolysis of hippurate ... ..	4 ,,

One mastitis strain was identical with the bovine salivary strain *Str. viridans bovis* (Belenky) in all its cultural characteristics.

No Edinburgh mastitis strain of viridans type corresponded exactly with the *Str. agalactiae* (Minett) strains in biochemical reactions, and hippurate-splitting strains were in a minority in contrast to the relatively constant hydrolysis of hippurate by the *Str. agalactiae* (Minett) strains.

Two of the viridans mastitis strains of our series were identical with the normal milk strain, failing to grow on MacConkey's medium and to hydrolyse hippurate. They both belonged to the biochemical type—non-haemolyticus III (Holman).

*$\alpha'$  (alpha dash) type* (see Table II)

Eleven of our mastitis strains produced the  $\alpha'$  type of lysis in unaltered ox-blood agar with absence of green coloration on heated blood. In addition, 18-hour serum broth cultures were haemolytic in fairly small doses. The criterion for identification of strains of the  $\alpha'$  type was the appearance in the blood agar medium of a zone of imperfectly lysed blood in which, with the low power of the microscope, corpuscles could be seen. This appearance, taken in association with the absence of green production in heated blood media suggested that the strains should be regarded as essentially haemolytic rather than of viridans type. For comparison, a weakly haemolytic strain from normal milk was included in the test series.

*Morphology and colonial appearances.* No significant differences were noted in the size and shape of individual cocci and in the length of chains produced by these  $\alpha'$  mastitis strains and the normal milk strain. In and on solid blood media they differed from the normal milk strain in yielding small, granular greyish white colonies and not colonies of the colourless dewdrop type.

*Bile resistance.* Nine of the eleven  $\alpha'$  strains grew on MacConkey's medium within 5 days, while the normal milk strain failed to grow within that period.

*Growth in phosphate bouillon.* All eleven strains produced a flocculent deposit with clear supernatant. The normal milk strain formed only a diffuse turbidity without a deposit.

*Heat resistance.* The eleven mastitis strains in this group and the normal milk strain failed to resist 60° C. for 15 min.

*Haemolytic action.* The eleven  $\alpha'$  mastitis strains and the normal milk strain caused complete lysis of the test volume of red blood cells with 1.0 c.c. of whole culture and partial lysis with 0.5 c.c.

*Biochemical reactions.* Assuming that the strains in this group could be regarded as haemolytic, nine belonged to the pyogenes type (Holman), one to the anginosus type and one to the haemolyticus I type. The normal milk strain belonged to the faecalis type.

*Aesculin fermentation.* All the mastitis strains of  $\alpha'$  type and the normal milk strain failed to blacken the aesculin-bile-salt medium.

*Changes in litmus milk.* Seven of the eleven strains acidified milk at 15° C. and two at 37° C. The other nine strains produced acid and clot in milk at 37° C. The normal milk strain acidified milk only at 37° C.

*End pH of glucose bouillon culture.* The eleven mastitis strains and the normal milk strain produced an end pH of 4.5 or less.

*Methylene-blue reductase production.* Only one of the eleven mastitis strains reduced the dye. The normal milk strain failed to do so.

*Hydrolysis of sodium hippurate.* Eight of the eleven mastitis strains hydrolysed hippurate. The normal milk strain failed to do so.

#### *Summary of $\alpha'$ (alpha dash) strains.*

Of the eleven mastitis strains, two were quite typical of the human haemolytic streptococcus except for weaker haemolysin production in fluid culture. The other nine strains were unlike *Streptococcus pyogenes* of man in the following respects:

Growth on MacConkey	...	...	...	...	9 strains
Hydrolysis of hippurate	...	...	...	...	8 „
Only partial lysis with 0.5 c.c. of fluid culture	...				11 „

Six strains in this group were identical with *Str. agalactiae* (Minett). They grew on MacConkey's medium and hydrolysed hippurate.

The strain from normal milk failed to grow on MacConkey's medium and to hydrolyse hippurate but belonged to the *Str. faecalis* biochemical type by Holman's classification.

*β (beta) type* (see Table II)

Six mastitis strains in our series yielded colony appearances on ox-blood agar and on the heated blood medium which suggested their classification as haemolytic. They showed a broad zone of complete lysis on the unaltered blood medium in association with absence of green pigmentation on heated blood. In addition, 18-hour serum broth cultures were haemolytic in small doses (0.1 c.c.). They were regarded as *Str. haemolyticus* and their properties were further examined. Four human haemolytic throat strains from scarlatina and two haemolytic strains of *Str. agalactiae* (Minett) were examined in the same culture media for comparative purposes.

*Morphology and colonial appearances.* No significant differences were noted in the size and shape of individual cocci and in the length of chains produced. In and on solid blood media five of the six haemolytic mastitis strains and the two *Str. agalactiae* (Minett) strains formed granular, greyish white colonies. One mastitis strain of this type and all four human haemolytic throat strains formed smaller, colourless colonies of the dewdrop type on solid blood media.

*Bile resistance.* Three of the six mastitis strains grew on MacConkey's medium within 5 days as did one of the two haemolytic strains of *Str. agalactiae* (Minett). The other strain of *Str. agalactiae* (Minett) and all four human haemolytic throat strains failed to grow on MacConkey's medium within 5 days.

*Growth in phosphate bouillon.* Five of the six  $\beta$ -haemolytic mastitis strains, two of the four human haemolytic throat strains and both the *Str. agalactiae* (Minett) strains produced a flocculent deposit and clear supernatant. The remaining mastitis strain produced a diffuse turbidity with no deposit and the other two human haemolytic throat strains formed a dense turbidity with a granular deposit.

*Heat resistance.* Only one of the six mastitis strains of  $\beta$  type resisted 60° C. for 15 min. All other strains failed to do so.

*Haemolytic action.* All six mastitis strains and both strains of *Str. agalactiae* (Minett) caused complete lysis of the test volume of red blood cells with 0.5 c.c. of whole fluid culture and partial lysis with 0.1 c.c. All four human haemolytic throat strains caused complete lysis with 0.1 c.c. of whole fluid culture.

*Biochemical reactions.* Four of the six  $\beta$ -type mastitis strains belonged to the *Str. pyogenes* biochemical type of Holman, one to the anginosus type and one to the infrequens type. All four human haemolytic throat strains belonged to the *Str. pyogenes* type as did one strain of *Str. agalactiae* (Minett). The other *Str. agalactiae* (Minett) strain belonged to the anginosus type.

*Aesculin fermentation.* Only one of the six mastitis strains blackened the aesculin-bile-salt medium. All other strains failed to do so.

*Changes in litmus milk.* Three of the six mastitis strains acidified milk at 15° C. and all six produced acid and clot in it at 37° C. The four human

haemolytic throat strains and the two strains of *Str. agalactiae* (Minett) only acidified milk at 37° C.

*End pH of glucose bouillon culture.* All six of the haemolytic mastitis strains and both *Str. agalactiae* (Minett) strains produced an end pH of 4·5 or less. All four human haemolytic throat strains attained an end pH of 4·5–5·5.

*Methylene-blue reductase production.* Two of the six mastitis strains reduced the dye and all other strains failed to do so.

*Hydrolysis of sodium hippurate.* Three of the six mastitis strains hydrolysed hippurate as did both haemolytic strains of *Str. agalactiae* (Minett). All other strains failed to do so.

#### *Summary of the $\beta$ (beta) strains.*

Of the six Edinburgh mastitis strains, two were quite typical of the human haemolytic streptococcus. The remaining four were atypical in the following respects:

Growth on MacConkey's medium ... ..	3 strains
Resistance to 60° C. for 15 min. ... ..	1 „
Fermentation of aesculin in bile salt medium ...	1 „
Hydrolysis of hippurate ... ..	3 „

Only one of the six haemolytic strains was identical with *Str. agalactiae* (Minett).

#### *$\gamma$ type (see Table III)*

Twenty Edinburgh mastitis strains were inert in both unheated and heated ox-blood agar. Two  $\gamma$  strains from human faeces and from normal milk as well as three  $\gamma$  strains of *Str. agalactiae* (Minett) and one from mastitis (received from Miss Anderson, Rowett Institute, Aberdeen) were examined in the same culture media for comparative purposes. The last strain is referred to as the MA strain.

*Morphology and colonial appearances.* Two of the twenty mastitis strains of our series, the two human faecal strains and one of the two normal milk strains were lanceolate diplococci, distinct from all other strains which appeared as rounded cocci in chains of variable length. The mastitis strains, the human faecal strains, the *Str. agalactiae* (Minett) strains, the MA strain and both strains from normal milk yielded larger and more opaque colonies than the common dewdrop type. Seven of the twenty Edinburgh mastitis strains, both human faecal strains and one of the normal milk strains produced a thick, greyish white growth on heated ox-blood agar.

*Bile resistance.* Thirteen of the twenty  $\gamma$ -type mastitis strains grew on MacConkey's medium and seven did not. Both human faecal strains, one of the two normal milk strains and two of the three *Str. agalactiae* (Minett) strains also grew on MacConkey's medium.

*Growth in phosphate bouillon.* Thirteen of the twenty mastitis strains, the two human faecal strains, one of the normal milk strains, the three *Str. agalactiae* (Minett) strains and the MA strain all caused a dense turbidity with

a heavy granular deposit. Five of the Edinburgh mastitis strains and the other normal milk strain produced only a moderate diffuse turbidity. The remaining two strains from the Edinburgh cases of mastitis formed a flocculent deposit with clear supernatant.

*Heat resistance.* Four of the twenty mastitis strains resisted 60° C. for 15 min. Both human faecal strains and one of the two normal milk strains resisted the same degree of heat. All the other strains were heat-sensitive.

*Haemolytic action.* All the  $\gamma$  strains tested failed to cause lysis of the test volume of red blood cells with 1.0 c.c. or smaller quantities of whole culture.

*Biochemical reactions.* Of the twenty  $\gamma$ -type mastitis strains, seven belonged to the mitis type of Holman, five to the ignavus type, three to the faecalis type, three to the non-haemolyticus I type and two to the salivarius type. Both human faecal strains and one of the two normal milk strains belonged to the faecalis type. The other normal milk strain belonged to the non-haemolyticus I type. All three strains of *Str. agalactiae* (Minett) were classified as the salivarius type and the MA strain belonged to the mitis biochemical type.

*Aesculin fermentation.* Only two of the twenty mastitis strains, both human faecal strains and one of the two normal milk strains blackened the aesculin-bile-salt medium. All other strains failed to do so.

*Changes in litmus milk.* Nine of the twenty  $\gamma$ -type mastitis strains, both human faecal strains and one normal milk strain acidified milk at 15° C. Nine mastitis strains, one of the two human faecal strains, the three *Str. agalactiae* (Minett) strains and the MA strain acidified milk at 37° C. Seven of the mastitis strains, the other human faecal strain and both normal milk strains produced both acid and clot in milk at 37° C.

*End pH of glucose bouillon culture.* Fourteen of the twenty mastitis strains in this group, both human faecal strains, both normal milk strains, the MA strain and two of the three *Str. agalactiae* (Minett) strains produced an end pH of 4.5 or less. Five of the mastitis strains and the remaining strain of *Str. agalactiae* (Minett) produced an end pH of 4.5-5.5. The remaining mastitis strain reached an end pH of more than 5.5.

*Methylene-blue reductase production.* Nine of the twenty mastitis strains, both human faecal strains and one of the two normal milk strains reduced the dye. All other strains failed to do so.

*Hydrolysis of sodium hippurate.* Six of the twenty mastitis strains, all three strains of *Str. agalactiae* (Minett) and the MA strain hydrolysed hippurate. All other strains did not cause hydrolysis.

#### *Summary of $\gamma$ (gamma) strains.*

Of the twenty  $\gamma$ -type Edinburgh mastitis strains, only one was quite typical of the human enterococcus. It was heat-resistant, blackened the aesculin-bile-salt medium and fermented mannite. The other nineteen strains were atypical of *Str. faecalis* of man in the following respects:

No growth on MacConkey's medium	...	...	...	7 strains
Killed at 60° C. in 15 min.	...	...	...	16 „
No fermentation of mannite	...	...	...	14 „
No growth in milk at 15° C.	...	...	...	11 „
No fermentation of aesculin in bile-salt medium...	...	...	...	18 „
No methylene-blue reductase produced	...	...	...	11 „
Hydrolysis of hippurate	...	...	...	6 „

One Edinburgh mastitis strain conformed to the characteristics of *Str. agalactiae* (Minett). One normal milk strain was identical with *Str. faecalis* and the other corresponded with one Edinburgh mastitis strain of the non-haemolyticus I type.

DIFFERENTIATION OF HUMAN FROM BOVINE STREPTOCOCCI BY  
FERMENTATION OF SORBITOL AND TREHALOSE

The work of P. R. Edwards (1932, 1933) and others has suggested that a clear-cut differentiation of human from bovine streptococci could be achieved by the use of sorbitol and trehalose. Human strains were shown to ferment trehalose but not sorbitol, while bovine strains fermented sorbitol but not trehalose. Owing to delay in obtaining a supply of trehalose these reactions were not studied in the case of all strains examined. Representative strains from bovine mastitis were, however, compared with a number of human strains with the results given in Table IV. It will be seen that fifteen out of twenty bovine strains failed to ferment sorbitol and eighteen out of twenty-three human strains failed to ferment trehalose. Observations were made daily for 5 days, different batches of the sugars were tested, and controls were employed with organisms known to ferment the carbohydrates. In addition, all tubes were tested for growth after 5 days by subculture on blood agar.

Table IV. *Human and bovine strains. Fermentation of sorbitol and trehalose*

Source of strain	Type on blood agar	No. of strains	Fermentation of sorbitol			Fermentation of trehalose		
			Positive	Doubtful	Negative	Positive	Doubtful	Negative
Mastitis	$\alpha$	6	0	4	2	5	0	1
„	$\beta$	7	0	1	6	6	0	1
„	$\gamma$	7	0	0	7	2	0	5
Scarlatina	}	23	0	1	22	4	1	18
Puerperal fever								
Tonsilitis								

DISCUSSION

Previous workers on the bacteriology of bovine streptococcal mastitis have classified the strains isolated by methods unfamiliar to medical workers. The latter, accustomed to a sharp differentiation into  $\alpha$ ,  $\beta$  and  $\gamma$  types according to the action upon blood, have been confused by a classification which ignored this criterion. The purpose of the present work was to examine mastitis strains

by the methods usually employed by medical bacteriologists in an endeavour to determine whether the streptococci isolated from bovine mastitis are similar to the haemolytic and non-haemolytic streptococci of human disease or not. The action upon blood was employed as the first basis of differentiation. Our mastitis strains could be grouped into types by this method but the other associated characteristics of the strains were quite unlike those of the corresponding types usually encountered in human infections.

Thus the  $\alpha$ -type strains from mastitis differed in several respects from the classical *Str. viridans* as found in the human throat. The majority of our mastitis strains grew on MacConkey's medium and fermented mannite but not raffinose, while some strains hydrolysed hippurate.

The haemolytic strains from mastitis were divisible into two groups—one producing strong haemolysis of the  $\beta$  type and the other weak haemolysis of the  $\alpha'$  type. Both groups differed from the corresponding type of human streptococcus in various ways. The haemolytic mastitis strains produced a more luxuriant growth on heated blood agar and formed weaker haemolysin in fluid culture. Most of these strains also grew well on MacConkey's medium and hydrolysed hippurate. A few mastitis strains were almost typical of *Str. pyogenes*, differing from it in only one of these respects, while four of the haemolytic strains from mastitis were quite typical of *Str. pyogenes*.

The mastitis strains of our series which were inert on solid blood medium were, with one exception, unlike the  $\gamma$  or enterococcus type from human faeces. Most strains failed to resist 60° C. for 15 min., to blacken the aesculin-bile-salt medium and to ferment mannite. These tests are all usually positive in the case of *Str. faecalis*. In addition, the  $\gamma$  strains encountered in udder infections were not uniform in bile resistance, production of methylene-blue reductase, growth in milk at 15° C. and hydrolysis of sodium hippurate.

From such a comparison it was concluded that the majority of the strains of streptococci from bovine mastitis differed in biological characteristics from the streptococci of human origin.

The second aim of the present work was to define the so-called "*Streptococcus mastitidis*"—the streptococcus concerned in the aetiology of the well-defined clinical condition of bovine mastitis—by the methods applicable to human streptococci. This attempt had results similar to those yielded by the biochemical and serological methods used by others. The mastitis strains showed a marked heterogeneity in their cultural and biochemical characteristics. When every test was taken into consideration, no two strains from bovine mastitis were found to be identical in biological characteristics. This lack of even approximate uniformity among strains is in contrast to the condition of affairs found in human specific diseases of streptococcal origin. In scarlatina, for example, the causative streptococcus is relatively uniform in its cultural characteristics. *Str. haemolyticus* of human scarlatina is invariably  $\beta$ -haemolytic, does not grow on MacConkey's medium, cannot resist 60° C. for 15 min., ferments lactose and salicin but not usually raffinose, mannite or inulin, does

not grow in milk at 15° C., does not produce methylene-blue reductase and does not hydrolyse hippurate. In direct contrast, the strains of streptococci isolated from the specific bovine clinical condition of mastitis were distributed over all four cultural types on blood media and exhibited a corresponding lack of uniformity in all the other tests.

Approximate uniformity in cultural and biochemical reactions was only observed among those strains from mastitis giving weak haemolysis in blood agar which we have classified as of  $\alpha'$  type (see Table II). In this group the great majority of strains had the same characteristics as those weakly haemolytic streptococci from bovine mastitis and the human vagina described in a recent paper by Hare and Colebrook (1934) with whom our results are in general agreement. It must be noted however that this group comprised only eleven of our fifty-one strains.

Our observations emphasise the difficulty of identifying a streptococcus as of bovine origin when it is encountered in human infections. Such well-recognised tests as hippurate hydrolysis, sorbitol fermentation and growth on bile media have been found negative in a large proportion of strains from cases of mastitis.

#### SUMMARY AND CONCLUSIONS

Fifty-one strains recently isolated from cases of bovine mastitis have been studied in their morphological, cultural and biochemical reactions.

Using their colony appearance in blood agar as the first differential criterion we have shown that the strains differ widely from one another and from the  $\alpha$ ,  $\beta$  and  $\gamma$  streptococci usually encountered in human disease.

The heterogeneity of mastitis streptococci was strikingly demonstrated. From the results of a relatively small number of cultural and biochemical tests it could be shown that no two of the fifty-one strains were identical, in every respect.

The findings recorded suggest that great caution must be exercised in ascribing a bovine or human origin to individual strains of streptococci on the results of cultural or biochemical reactions.

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