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Streptococcus uberis and its ecology in the dairy cow

Radhey Mohan Sharma

Iowa State University

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Iowa State University, Ph.D., 1969
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STREPTOCOCCUS UBERIS AND ITS ECOLOGY IN THE DAIRY COW

by

Radhey Mohan Sharma

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major Subject: Veterinary Microbiology

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Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Ames, Iowa

1969
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INTRODUCTION

Streptococci are an important cause of bovine mastitis, a disease of great economic importance to the dairy industry throughout the world. Maximum attention has been concentrated on the study of *Streptococcus agalactiae* and its control in milk cattle, and some success has been achieved. *Streptococcus uberis*, as a cause of bovine mastitis, did not receive adequate attention in the past. Work carried out during the last few years has shown that it is frequently associated with bovine mastitis and is an organism of great importance to the dairy industry. The incidence of *Str. uberis* infection appears to have increased as reported by Hughes (1960). Arthur (1947) and Animal Health Report of Great Britain (1957) gave figures of approximately nine per cent *Str. uberis* infection in cows. Stableforth (1953) mentioned an incidence of 12.0 per cent *Str. uberis* infection as a cause of clinical mastitis in 1952, based on the examination of 2005 milk samples. Hughes (1960) reported that *Str. uberis* comprised 18.0 per cent of all infections responsible for the causation of mastitis and that it was second to *Str. agalactiae* in numerical importance. Edwards and Smith (1966) studied the epidemiology of mastitis in three herds in England for 14 years and reported that 315 samples out of 1535 (20 per cent) had *Str. uberis* infection.

The published work on *Str. uberis* shows inconsistencies, and the complete definition, and exact delineation of the species has not been made clear. There has been much disagreement on the biochemical properties of *Str. uberis* and its serological classification has not yet been fully
elucidated. Many strains of *Str. uberis* do not react with any of the Lancefield group antisera. Only 20 to 21 per cent of them have been reported to belong to serogroup E, but they do not induce group E antibodies in rabbits. No attempt seems to have been made to study *Str. uberis* bacteriophages with a view to explore the possibility of developing a system of phage-typing of this organism.

The findings reported by research workers on the ecology of *Str. uberis* in the cow also differ. Sweeney (1964) mentioned that the skin of the udder accounted for 73 per cent of the isolations of this organism and that infection of the mammary gland parenchyma was secondary to infection of the udder skin. Cullen (1966), however, reported that different skin sites had extremely varied isolation rates, the belly and lips were most heavily infected. He added that though skin infection was widespread at times, the skin of the teats was always a comparatively unfavorable site for *Str. uberis*, the lips were the best site for its survival.

In view of the diversity of results reported on the biochemical and serological reactions and the ecology of *Str. uberis*, it was considered desirable to carry out a detailed investigation into its ecology in the dairy cow, and to study its biochemical and physiological characters with a view to characterize it more completely. A study of the ecology seemed necessary to gain more information as it forms an important basis to devise suitable measures for the control of this infection in cows. The control measures can be conveniently employed if udder and teat surfaces constitute the main source of infection of the mammary gland parenchyma. If, however,
the skin remote from the udder is more heavily infected and it contributes to the infection of the mammary gland, a method aimed to ensure disinfection of the udder and teat skin would do little to reduce total infection of the skin by *Str. uberis* throughout the herd. Neither is the successful treatment of infection with intramammary administration of antibiotics likely to prevent further sporadic cases from occurring in the herd as the source of infection would continue to persist on the body surface.
REVIEW OF LITERATURE

Characterization of *Streptococcus uberis*

*Streptococcus uberis* is an important organism because of its frequent association with bovine mastitis. It has rarely been isolated from species of animals other than cattle and has not been incriminated as a pathogen of man. Ayers and Mudge (1922) examined 100 strains of streptococci selected at random from udders, most of which were healthy, though a few were affected with mastitis. Seventy-nine strains fell into a closely defined group which they believed to be identical with *Streptococcus mastitidis*, and they were able to divide it further into sub-groups A, B, C, and D. Among the other isolates, their groups E and F (3 and 2 strains respectively) agreed with the characters now recognized as those of *Streptococcus uberis*, although they did not give them a name. Grini (1944) also noted the similarity between group E of Ayers and Mudge and *Streptococcus uberis*, and considered that these authors were the first to describe the organism. However, it was not before 1928 that these less common mastitis pathogens were closely defined.

Klimmer *et al.* (1928) described 321 strains of streptococci isolated from milk. They placed them in five groups, of which group 1 was the largest, containing 201 strains of *Streptococcus agalactiae*. Group 2 contained 21 strains which in retrospect seem likely to have been *Streptococcus uberis*.

Diernhofer (1932) reported isolation of atypical strains of streptococci from cases of mastitis in cows which he designated as *Streptococcus uberis*. Edwards (1932) found that a mild form of mastitis, transient in its...
course, or at times tending to be somewhat chronic, might be caused by a species of streptococcus other than those previously reported by Minett et al. (1929, 1932). Edwards (1932) designated these non-hemolytic streptococci as mastitis streptococcus group III, as distinct from mastitis streptococcus groups I and II. Diernhofer (1932) reported that the streptococcal strains, isolated by him from milk from infected udders, were found to be identical with mastitis streptococcus group III (Edwards, 1932). Diernhofer confirmed the majority of results reported by Edwards (1932) and applied additional tests. He described the characters of the species as: smooth, round colonies on agar; turbidity in broth; diplococci; litmus milk gradually reddened, no coagulation; no change in litmus milk at 10°C. with a scanty inoculum; transient reduction of methylene blue milk, no coagulation; acid formation from glucose (final pH 4.7), lactose, sucrose, mannitol, and salicin, but not from raffinose and glycerol. Sodium hippurate and esculin were split; sugar-free blood agar plates showed greening in deep colonies. Diernhofer indicated that this organism also possibly corresponded to certain exceptional mastitis strains described in Germany by Klimmer et al. (1928). Diernhofer (1932) proposed the species name Streptococcus uberis. Minett (1934), however, did not agree to giving a specific name of Str. uberis to this organism and suggested that pending international agreement, it might be called mastitis streptococcus group III. The principal reactions of the organism as reported by him were: non-hemolytic; slight diffuse growth in broth resulting in turbidity; acid or acid and soft clot, partial reduction of litmus milk at 37°C; production of slight acidity at 10°C;
growth and reduction or reduction and coagulation of milk containing 1:20,000 methylene blue; final pH of 4.7 to 4.9 in 1 per cent lactose broth; fermentation of salicin, mannite, inulin and esculin, but not raffinose; and hydrolysis of sodium hippurate.

Plastridge et al. (1934) classified 208 strains of mastitis streptococci in which the group designated "Ba" was later found to agree with the description of Str. uberis. Plastridge (1939) compared his group "Ba" streptococci with a strain of Str. uberis of Diernhofer and found them to be in agreement, and he adopted the name "Str. uberis" for his group "Ba cultures".

Brown (1937) described streptococci of Lancefield's groups A, B, C, D, E, F, G, H, and K which he had isolated from human, equine and bovine sources. His three group E strains appear to have been Str. uberis, and were derived from cattle. He mentioned that they were not found in man, and considered them to be of doubtful significance. Miller and Heishman (1940) found the organism extremely pathogenic. They classified 69 strains of streptococci obtained from a herd which had been troubled by persistent mastitis. They defined five groups, on the basis of 14 physiological tests, including precipitation with Lancefield's group-specific serum. Their group 5 was Str. uberis and was the only one which had been causing mastitis. Little (1940) found Str. uberis as a persisting infection on two or more monthly inspections in three out of four herds. He outlined the cultural characters of Str. uberis.

Stableforth (1932) studied the serological characters of mastitis
streptococci and reported that the precipitation test was applicable to all types of streptococci examined, except those of group III, which appeared to be deficient in type-specific protein. The results obtained by precipitation and agglutination were generally found to be parallel in the case of groups I and II, but not in case of group III. Of the 40 strains of group III, only 15 could be classified, the remaining 25 gave negative results with all sera used by him. Plastridge and Hartsell (1937) examined 19 strains of their "Ba cultures" (apparently Str. uberis) by the precipitation method and reported three types containing 6, 4, and 3 strains, respectively. Plastridge and Williams (1939) examined 141 strains by the agglutination method and found that 136 fell into 11 types, whilst 5 were negative with all sera. "Whilst the majority of the cultures were type-specific, about 20 per cent shared a common antigen with more than one serological type." Little (1940) also recorded three types, whilst Slanetz and Naghski (1940) reported the finding of strains that fell into two of Plastridge and Hartsell's (1937) types. Little et al. (1946) mentioned that the serological identification of Str. uberis and the place it should occupy in Sherman's classification (1937) had not been definitely determined. A total of 277 biochemically similar strains were examined and were placed in 15 serological types by the precipitation and agglutination methods. About one-third of the cultures cross-agglutinated with 2 to 5 antisera. A somewhat wider range of cross-reactions was obtained by the precipitin method. A few strains gave cross-agglutination and precipitin reactions with group E antiserum, but reciprocal agglutinin absorption tests
showed that such cultures were not serologically identical with Lancefield group E (type culture K 129). About 10 per cent of the strains studied by them, although possessing many of the cultural characteristics of Strep. uberis, were negative in inulin; however, such cultures were serologically identical with some inulin-positive strains. The above authors indicated that though the physiological properties of Strep. uberis were not identical with those of any of the species described by Sherman (1937), they resembled the general properties of enterococcus division more closely than those of any of the other groups described by him. Slot (1958) carried out a detailed study of 156 strains of Strep. uberis. He applied 27 different physiological tests, and concluded that the fermentation reactions were quite uniform. He added that the organism did not fit exactly into any of the Sherman groups. The serological relationship of some strains to group D was mentioned (Sherman, cited by Little et al., 1946) but it has not been clearly demonstrated, and in fact has been contradicted (Jacob, 1947).

The published work on Strep. uberis shows inconsistencies, and the complete definition and exact delineation of the species has not been made clear. The serological classification of Strep. uberis has not yet been finally elucidated, and there has been much disagreement on the biochemical properties of the organism. Efforts to place Strep. uberis in existing classifications of the streptococci on a physiological basis have resulted in differences of opinion. Plastridge and Williams (1939) expressed the view that Strep. uberis bore a closer physiological resemblance to Lancefield's
group E than to any other group. Slanetz and Naghski (1940) mentioned that \textit{Str. uberis} resembled the enterococci in many characteristics. Hansen (1935) observed that \textit{Str. uberis} might form a transitional type between \textit{Str. agalactiae} and the fecal streptococci. Extracts of some \textit{Str. uberis} react in some group E sera but do not induce group E antibodies in rabbits (Minett and Stableforth, 1934; Bliss, 1937; Thal and Moberg, 1953; Jacob, 1956). Sweeney (1964) reported that 21 per cent of the strains of \textit{Str. uberis}, isolated from udder surface, and 16 per cent of those from milk samples of cows at Weybridge, England, reacted with group E antiserum.\(^1\)

He added that a similar proportion of sero-group E strains were encountered among the reference collection from commercial herds, suggesting that the strains infecting the Weybridge herd might be representative of those in many other herds. Heeschen and Meyer (1965) found that 41 out of 46 strains reacted with group E serum but the source of their serum was not stated. Cullen (1966) mentioned that 83 of 486 strains of \textit{Str. uberis} (17.10 per cent), isolated from skin sites on the body and milk samples of cows, reacted with group E antiserum. He added that 13 strains which proved sorbitol-negative and four isolates that fermented raffinose, also reacted with group E antiserum. Later Cullen (1967) published results of the study on 1481 strains of the organism and suggested that inulin-negative strains should not be included in the \textit{Str. uberis} species. He described a number of

intermediate types, linking *Str. uberis* with *Str. faecalis*, and proposed that this organism should be placed in the enterococcus division of Sherman (1937), rather than the viridans or pyogenes group.

In view of the diversity of opinion expressed in regard to some of the biochemical and physiological reactions of *Str. uberis*, it seems desirable that a summary of the results reported by different workers may be tabulated in respect to the more important characteristics.

**CAMP reaction**

Christie et al. (1944) described a test for the identification of *Str. agalactiae* and reported that it was found accurate to identify 200 strains of group B streptococci, and that 395 strains from other Lancefield groups and 20 ungrouped strains comprising *Str. uberis* and *Str. dysgalactiae* failed to induce the lytic phenomenon. Munch et al. (1945) mentioned that streptococci other than group B either did not produce the filtrable thermostable agent which lysed sheep or ox red cells in the presence of staphylococcus beta toxin or did so to a negligible degree. Munch-Petersen and Christie (1947) reported that the *Str. agalactiae* colonies lysed the red cells in less than two hours to a distance of 10 mm. within the area covered by the staphylococcal beta toxin. Wilson and Slavin (1950) mentioned that they could not discover a group B streptococcus which failed to produce the completion of lysis in the beta toxin zone but that 10 to 20 per cent of strains of *Str. uberis* also exhibited this reaction. Murphy et al. (1952) reported that of 322 cultures classed as *Str. uberis* (principally because of ability to split esculin), 222 failed to
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react to CAMP test; 39 produced some degree of reaction. Of 46 cultures classed as *Str. dysgalactiae*, none produced a CAMP reaction. They added that the presence of 0.1 per cent esculin in the blood agar significantly enhanced the intensity of the reaction, but did not significantly affect the growth of the streptococci. The presence in the blood agar of ferric citrate (10 ml. of 1 per cent aqueous solution per liter) did not affect either the growth or the intensity of the reaction, but did significantly improve the ability to detect splitting of esculin when it occurred. Fey (1953) subjected 384 strains of streptococci to the CAMP test and reported that of the 200 strains of group B known to be *Str. agalactiae*, 198 were CAMP-positive. Three strains not belonging to group B were also found positive to the CAMP test. Hauge and Ellingsen (1953) found that certain strains of *Str. uberis* gave a zone of hemolysis similar to that produced by *Str. agalactiae* on a modified Edwards' medium to which 4-6 per cent staphylococcal beta toxin had been added. Slot (1958) studied the biochemical and physiological reactions of 156 strains of *Str. uberis*, 134 of them were CAMP-positive. Heeschen and Meyer (1965) reported that nine of the 46 strains, examined by them, showed CAMP-positive reaction. Batis and Brulez (1964) mentioned that three strains of *Str. dysgalactiae* also gave positive CAMP reaction. Postle (1968) reported that CAMP-positive streptococci isolated from bulk milk on TKT (Thallium, crystal violet, toxin) medium were classified as either *Str. agalactiae* or *Str. uberis* on the basis of the CAMP reaction and the esculin splitting ability of the organisms. It was found that 87 per cent of bulk milk samples from a limited area contained CAMP-positive streptococci and of this group, 98 per cent contained both *Str. agalactiae* and *Str. uberis*. 
Bacteriophages of Streptococci

There are many reports on the bacteriophages of hemolytic streptococci. Considerable literature is available concerning the bacteriophages of lactic streptococci. Work on the bacteriophages of group D streptococci has also been reported by some workers.

Evans (1934) described four streptococcus phages, designated A, B, C and D. Their distinct behavior in cross serological reactions was the only character which differentiated them. She examined the 4 races of phage in the nascent state for their ability to attack 421 strains of hemolytic streptococci. Phage A lysed 89.3 per cent of strains, phage B lysed 88.4 per cent, phage C lysed 79.3 per cent, and phage D lysed 9.7 per cent of the strains. She found that pneumococci were more sensitive than hemolytic streptococci to the four types of phage in the nascent state. None of the few strains of the alpha type of streptococci examined was sensitive to the phage. A strain of *Streptococcus lacticus* was found sensitive to phage D, and one of a few strains of staphylococcus examined was sensitive to phage A. Evans and Sockrider (1942) isolated another distinct phage active against Lancefield's group A streptococci. This phage was specific for the lactose-deficient and mannitol-fermenting sub-groups of Lancefield's group A streptococci (Evans, 1941). McKenna (1952) isolated another phage from sewage in an attempt to type beta-hemolytic streptococci with phages. Kjems (1955) described a technique for isolating streptococcal phages from phage-producing cultures. He reported the action of four different types of phage upon hemolytic streptococci of group A and
the range of activity and specificity of the phages. He added that streptococcal phages might afford a means of differentiating between strains of streptococci that were closely related serologically. Kjems (1960b) and Krause (1957) confirmed the specificity of group boundaries with phages active on group A streptococci. On the other hand, Maxted (1955), using group A phages identical to those used in Kjems's studies, did not find the phages specific for their propagating strains.

Bacteriophages for lactic streptococci have been known since the early work of Whitehead and Cox (1935). Since then these phages have been the subject of many research investigations ranging from growth and nutritional studies to studies on means of controlling their lytic action in dairy fermentation. Lactic phages have been isolated from whey samples collected from various dairy plants (Deane and Nelson, 1952) and from cheese milk failing to coagulate because of apparent phage contamination (Henning et al., 1964).

Baggar (1926) was perhaps the first to describe bacterial viruses that attacked group D streptococci. Houston (1936) mentioned that an active enterococcal phage could often be isolated from the stools in cases of ulcerative colitis. Graham and Bartley (1939) studied three phages specific for enterococci. Evans and Chinn (1947) indicated that enterococcal bacteriophages might be more widespread than phages attacking other streptococci. Brock (1964) isolated five other group
D streptococcal phages active against \textit{Str. faecalis}, \textit{Str. faecalis} var. 
zymogenes, and \textit{Str. faecalis} var. liquefaciens. Phages active against 
group D streptococci were also isolated by Bleiweis and Zimmerman 
(1961), Ciuca et al. (1959) and Rogers and Sarles (1963). Brailsford 
and Hartman (1968) reported isolation of \textit{Streptococcus durans} bacterio-
phages from bovine rumen-fluid, feed lot soil, bovine fecal samples, 
and lysogenic strains of \textit{Str. durans}. The presence of different phage 
strains was suggested by antiserum neutralization tests and one-step 
growth characteristics, whereby the bacteriophages were divided into 
three distinct, but serologically related groups. Kjems (1955) noted 
group specificity for phages active on the enterococci. Brock (1964) 
did not, however, find any degree of group specificity for the entero-
cocci, but he did find a high degree of strain specificity in \textit{Str.} 
faecalis and \textit{Str. faecium}. Ciuca et al. (1959) mentioned that enteroc-
coccal phages displayed a specificity for various species of group 
D streptococci.

Lysogeny among streptococci has been reported by various workers. 
Krause (1957) examined 57 strains of group A streptococci and found that 
12 of them were lysogenic. Kjems's study (1960a) reflected the extent of 
lysogeny in beta-hemolytic streptococci. He examined 353 isolates from 
throat swabs of patients and found 28 per cent of them lysogenic when
tested with a single indicator strain. Brock (1964) reported that there was a fairly high rate of lysogeny in Lancefield group D streptococci.

Although bacteriophage studies on streptococci were initiated in 1926, no report seems to be available on the phage-producing ability or phage sensitivity of Str. uberis. In view of the fact that the published work on the biochemical reactions of Str. uberis shows inconsistencies and its serological behavior is not yet fully elucidated to classify it appropriately, it was considered desirable to study the host-phage relationship in order to explore if a system of phage typing of this organism could be evolved.

**Incidence of Str. uberis**

*Streptococcus uberis*, as a causal organism of bovine mastitis, did not receive adequate attention in the past. Little et al. (1946) mentioned that Str. uberis was second in importance to Str. agalactiae in the causation of streptococcal bovine mastitis. Work carried out recently in Great Britain also indicated that it was the second most frequent cause of bovine mastitis. Infection with Str. uberis usually causes a mild clinically chronic form of mastitis but may cause severe damage to the affected quarter, especially if accompanied by obvious teat injury (Plastridge, 1953). It may be associated with an acute mastitis which later becomes chronic (Merchant and Packer, 1967). Str. agalactiae received maximum attention in the control of bovine mastitis in various countries and some success has been re-
ported in this behalf. The incidence of *Str. uberis* infection has been reported to vary in different countries. Watts (1951) mentioned that the incidence of *Str. uberis* infection in Ayrshire in 1939 was 2.3 per cent as compared to 33.5 per cent of *Str. agalactiae* and 4.3 per cent of *Str. dysgalactiae*, based on a study of 2699 cows from representative herds tested in toto. Slanetz and Naghski (1940) reported that of 680 cultures of weakly hemolytic streptococci, isolated from cows showing evidence of mastitis infection, 83 or 12.2 per cent were identified as *Str. uberis*, 573 or 84.2 per cent as *Str. agalactiae*, 15 or 2.2 per cent as *Str. dysgalactiae*, and 9 or 1.3 per cent as *Str. fecalis*. Jacob (1941) studied 240 strains of streptococci obtained from the udders of 94 cows from different parts of England. He classified 64 (26.8 per cent) as *Str. uberis*, 140 (58.0 per cent) as *Str. agalactiae*, 10 (4.16 per cent) as *Str. pseudo-agalactiae*, 8 (3.75 per cent) as *Str. dysgalactiae*. Van Der Scheer (1942) reported an incidence of 6.5 per cent *Str. uberis* infection in cows in Germany as revealed in a study on 1092 milk samples which proved positive for streptococcal infection. Ferguson (1943) conducted a bacteriological study of the role of udder injuries in establishing various infections of bovine mastitis at the Cornell University and found that of the 283 quarters, infected following trauma, *Str. uberis* was present in 43 or 15.0 per cent of the cows, *Str. agalactiae* in 67 (24.0 per cent), and *Str. dysgalactiae* in 65 (23.0 per cent) of the cases. In a previous study at Cornell, Ferguson (1938) reported an incidence of 10.8 per cent *Str. uberis* infection, based on a study of 229 cultures, isolated from quarter milk samples of
Neave et al. (1944) investigated an outbreak of clinical mastitis involving 26 cows in a herd of 60 animals believed to be free from Str. agalactiae. They did not find streptococcus group B organisms from any of these clinical cases. Eighty-two per cent of the affected quarters yielded large numbers of Str. uberis in their milk. Grini (1944) found Str. uberis (group III) associated with acute mastitis and in a couple of cases was the cause of serious attacks. He indicated that it appeared from Ayers and Mudge's description that their group E showed great resemblance to group III and it was believed to be identical with Str. uberis. Mohan (1945) isolated Str. uberis from cases of mastitis in cows at Mukteswar in India. Packer (1947) examined 2296 milk samples received from 60 practicing veterinarians in Iowa. He found 791 of them to contain mastitis organisms. Str. uberis was isolated from 54 milk samples against 57 positive for Str. agalactiae, and 30 which contained Str. dysgalactiae. The remaining milk samples were infected with other causal organisms. Rømer (1949) described findings of a survey of mastitis, conducted in Denmark on 298 herds, comprising 9916 cows. The percentage of infection was found to be 32.6 for cows with streptococci of group I (Str. agalactiae), while it was 1.9 for group II (Str. dysgalactiae), and 3.1 for group III (Str. uberis). The incidence of Str. uberis infection has also been reported from Pakistan (Rahman, 1963), Yugoslavia (Batis and Brglez, 1964), and Czechoslovakia (Gancić and Nemes, 1966).

The incidence of Str. uberis infection appears to have increased in
some countries during the last few years as reported by Hughes (1960). Arthur (1947), and Animal Health report of Great Britain (1957) gave figures of approximately 9 per cent *Streptococcus uberis* infection in cows there. Stableforth (1953) mentioned an incidence of 12.0 per cent *Streptococcus uberis* as a cause of clinical bovine mastitis in 1952, based on the examination of 2005 milk samples. Hughes (1960) reported that *Streptococcus uberis* was responsible for 18.0 per cent of all udder infections and that it was second to *Streptococcus agalactiae* in numerical importance. In 1960, a study was made of 143 herds, comprising 5000 cows (Wilson, 1963), with a view to determine the presence of infection whether or not accompanied by clinical signs. In 54 per cent of the samples, no significant bacterial growth was obtained. *Streptococcus uberis* was isolated from 7.5 per cent, *Streptococcus agalactiae* from 8.7 per cent, *Streptococcus dysgalactiae* from 4.5 per cent, and *Staphylococcus aureus* from 17.9 per cent. As a percentage of the number of samples from which organisms were isolated, the figure for *Streptococcus uberis* came to 16.4 per cent. Mück (1963) examined 416 cows in Germany and reported 77 cases of acute mastitis amongst them. Six cases (7.8 per cent) had *Streptococcus uberis* infection and four cows (5.2 per cent) were infected with *Streptococcus agalactiae*. A survey, carried out in India under the auspices of the Indian Council of Agricultural Research, revealed an incidence of 6.7 per cent *Streptococcus uberis* infection in cows and 17.2 per cent in buffalo cows (Dhanda and Sethi, 1962). Heeschen and Meyer (1965) reported 46 (23.7 per cent) strains of *Streptococcus uberis* from 194 cultures of streptococci isolated from 5000 milk samples from 550 cows. Edwards and Smith (1966) studied the epidemiology of mastitis in three dairy herds in England for 14 years and reported that 315 milk samples out of 1535 (20
per cent) yielded *Streptococcus uberis*. Walsh and Neave (1968) conducted a study on eight commercial herds of Friesian cows in Ireland and reported that the incidence of *Streptococcus uberis* infection as a cause of mastitis was 10.0 per cent.

*Streptococcus uberis* infection responsible for mastitis has also been reported in sheep. Pisanu and Manca (1964) gave an account of mastitis caused by *Streptococcus uberis* in 21 per cent of sheep in two flocks shortly after the introduction of machine milking. One-half of the udder was usually affected with permanent loss of function. *Streptococcus uberis* was isolated from milk and udder lesions.

**Ecology of Streptococcus uberis**

Slanetz and Naghski (1940) examined post mortem the udders of five cows which had been sampled at three-monthly intervals for three lactations, and had always appeared free from infection. They found *Streptococcus uberis* in two of these, but no other mastitis pathogens were present. Neave and Oliver (1962) cultured *Streptococcus uberis* from the teat orifice of six out of 20 heifers sampled 21 days after the end of their lactation. They believed that the animals must have become infected from their environment since they had not found any infection with *Streptococcus uberis* when samples were taken on the day of "drying off". Sweeney (1964) conducted studies on a laboratory herd of cows at Weybridge during a complete lactation. He reported that the skin of the udder accounted for 73 per cent of the isolations of this organism. The frequency of isolation from the udder surface was always higher than from within the mammary gland and infection of the former site
was sometimes found to be independent of the latter. Cullen (1966), however, mentioned that the skin was the most important reservoir of infection, but different skin sites had extremely varied isolation rates, the belly and lips were most heavily infected. He added that though skin infection was widespread at times, the skin of the teats was always a comparatively unfavorable site for \textit{Str. uberis}, the lips were the best site for its survival. Mammary gland infection was secondary to skin infection and \textit{Str. uberis} was rarely detected in feces. Cullen and Herbert (1967) mentioned that \textit{Str. uberis} was isolated more frequently from milk and skin swabs than from the teat canal in a study carried out on 7500 samples from 13 cows.

\textit{Str. uberis} has also been isolated from other sites apart from milk or intramammary tissue. Francis (1941) examined the tonsils from 100 slaughtered cattle and vaginal swabs of 40 dairy cows. \textit{Str. uberis} was isolated from seven pairs of tonsils, and appeared to be predominant organism in four cases. It was also isolated from one vagina. Grini (1944) isolated group III organisms (\textit{Str. uberis}) from the cervix uteri of a cow. Obiger (1954) reported isolation of \textit{Str. uberis} from the tonsils of 12 cattle out of 58 examined. Fornoni (1958) isolated \textit{Str. uberis} from the tonsils of one out of 222 cattle. Slot (1958) demonstrated the occurrence of \textit{Str. uberis} in the intestinal flora of cattle. He isolated four strains of \textit{Str. uberis} from 119 samples of feces from cows and calves. Winkenwerker (1966) reported isolation of \textit{Str. uberis} and other non-hemolytic streptococci
from the sexual organs of cattle and suggested that these organisms might enter the sexual organs as saprophytes and exert a pathogenic effect only during weakened resistance of the host animal. Cullen and Little (1968) isolated *Str. uberis* from the rumen of five out of 18 cows in a herd known to be infected with *Str. uberis*. Samples of soil taken from the field grazed by the herd, on examination, yielded *Str. uberis* seven times from 50 samples. These strains were identical to strains isolated from clinical cases.

**Relationship between age and lactation, and incidence of mastitis**

Davis and McClemont (1939) mentioned that the incidence of mastitis increased with age. Similar findings were reported by Priestley and Artioli (1945) based on the examination of 3322 milch buffalo-cows maintained at five military dairy farms at Lahore in West Pakistan. Murphy (1947) mentioned that the infection rate was shown to be a function of age and was unrelated to external factors, such as, milking-technique or management. Lancaster and Stuart (1951) reported that the susceptibility to infection of the bovine udder with *Str. agalactiae* was shown to be dependent mainly on the number of previous lactations and on previous infections. Spencer and Kraft (1949) described findings of a study conducted on mastitis in 12 herds. They mentioned that the degree of exposure to infection and other factors were more important than the age factor described by Murphy (1947). Seelemann (1932) and Livoni (1955) noted a direct relationship between age and *Str. agalactiae* infection. Seelemann (1932) reported an incidence of 8.6, 30.0, 42.0, 44.0, 52.0, and 52.0 infections in the 2-3, 3-4, 4-5, 5-6,
b-7, and 7-8 year age-groups of cows, respectively. Livoni (1955) found an incidence of 10.7, 21.9, 31.8, 35.7, 43.8, and 39.8 infections in the different age-groups mentioned above. Hughes (1960) observed that the distribution of Str. uberis infections resembled that of Str. agalactiae infections. He reported an incidence of 25, 45, 54, 86, 57, and 23 Str. uberis infections in cows in the various age-groups stated above, the peak incidence of 86 cows was in the 5-6 year age-group. Stableforth (1959) mentioned that most workers had noted a direct relationship between age and infection. There was some difference of opinion whether the high proportion of older cows found to be infected was a function of total duration of exposure or due to an increased susceptibility. The work of Lancaster and Stuart (1949, 1951) and Stuart and Lancaster (1949) suggested that older cows were in fact more susceptible. Murphy (1945) gave reasons for believing that a change in the tissues was responsible and he (1946) proposed an 'age-factor' for the factor concerned. Ormsbee and Schalm (1949), however, could not confirm this view and concluded that 'excluding cows with incompetent sphincter mechanisms, susceptibility is randomly distributed, regardless and independent of age and previous history of infection.' Stableforth (1959) mentioned that he agreed with Plastridge (1953), Ormsbee and Schalm (1949), and Spencer and Kraft (1949) in that whilst teat factors, antibodies in milk and tissue factors probably played a part in regard to susceptibility and might be associated with age, the factors of greatest importance were degree and duration of exposure as influenced by prevalence within the herd and hygienic measures taken to prevent infection. McEwen and Samuel (1946) carried out studies on the experimental bacterial contamination of
the inner structure of the teats and reported that it occurred in many cases when the outside of the teats was exposed to heavy bacterial contamination during either hand or machine milking. Edwards and Smith (1966) reported that the incidence of mastitis rose with increasing number of lactations and was prevalent in all cows, examined by them, in their sixth and subsequent lactations.

**Seasonal variations in incidence**

Hughes (1960) mentioned that *Staph. uberis* infections occurred most frequently in the winter months, and by the second half of the summer were 10 per cent or less of the total infections. There was some variation in this pattern from year to year, but broadly speaking the percentage distribution curve was U shaped, the arms of which were a reflection of the winter incidence of these infections. Priestley and Artioli (1945) reported that animals were susceptible to mastitis in early lactation, as judged by incidence figures. Watts (1951) divided the lactation period of cows into three parts and reported that of 369 infected cows, 157 showed the infection during the first three months, 82 during the second three months and 130 during the last quarter of lactation. The curves drawn from the figures for calvings for two years at 30 farms studied, gave two peaks, one in October and one in March. The period from August to November represented the period of greatest susceptibility to mastitis in mature cows and in recently calved heifers while the three months of December, January and February was the time that the majority of infected cows (that is, old cows) were dry. He, therefore, expected a low incidence of mastitis
in the first quarter of the year rising to a maximum toward the end of the year. Ineson and Cunningham (1949) mentioned that the number of new *Str. agalactiae* infections found during the spring, summer, fall, and winter were 54, 45, 75, and 38 respectively. The peak months of the year were October and April. Edwards and Smith (1966) reported that mastitis was of greater frequency in the spring and winter than in the summer. Cullen (1966) reported that maximum number of *Str. uberis* isolates could be obtained during the October-December quarter followed by the April-June quarter, then January-March period, and the least number of isolates were obtained in the July-September quarter. The greatest total of isolates for any one month was in May. This was not entirely in accord with the hypothesis that infection was best able to persist in the cold months of the year. He indicated that the stage of lactation had also been correlated with the apparent seasonal trends (Hughes, 1960). He thought that both weather and lactation stage might be important determinants of *Str. uberis* populations.
MATERIALS AND METHODS

Survey

Experimental herd

Twenty-four Holstein cows of the Dairy Farm, Iowa State University, were included in this study.

Herd samples

The herd of 24 cows was divided into two equal groups for the purpose of the study, each group comprised of 12 cows that were sampled on alternate weeks. The sites sampled were: right side of the udder, left side of the udder, teat surfaces, lips, nostrils, posterior part of the vagina, posterior part of the rectum, poll, side of the chest, sacral region (a few inches in front of base of tail, in mid-line), caudal folds, and belly (halfway between xiphoid and umbilical regions).

A sterile swab of cotton was dipped in sterile tryptose broth, the skin site was gently brushed and the swab was returned to the sterile glass tube. The milk samples were taken after the udder was washed with warm water and dried by the dairy man, the tip of the teat was cleaned with a pledget of cotton, soaked in ethyl alcohol. Composite milk samples were collected from the lactating quarters.

Culture media

Edwards (1933) took advantage of the fact that streptococci were more resistant to the antibacterial action of crystal violet than the majority of milk saprophytes. He devised a selective medium which afforded satis-
factory results in the cultural diagnosis of mastitis streptococci. It consisted of meat extract, crystal violet, defibrinated ox blood and esculin. This medium was used for the direct cultivation of milk samples collected from the cows under study. It was observed that gram-negative contaminants, particularly *Pseudomonas*, appeared in the plates. Packer (1943) reported that crystal violet used in a concentration of 1 in 500,000 and 1 in 200,000 in combination with 5 per cent blood agar failed to inhibit gram-negative rods. He added that the addition of sodium azide 1 in 2,000 to 5 per cent blood agar-crystal violet (1 in 500,000) medium, pH 6.8, helped to inhibit all the gram-negative contaminants. A small trial was carried out to study the effect of incorporating sodium azide in the Edwards medium in a concentration of 1 in 2,000 as compared to the original Edwards medium.

A second modification made was the substitution of esculin with inulin to detect inulin fermenting *Streptococcus* colonies.

The cultures of the test organisms were grown in tryptose broth, incubated at 37°C. for 18-24 hours. The 18-24 hour broth cultures were diluted in sterile saline immediately before inoculation into the media. In view of the great variation in the amount of growth of the different organisms at 18-24 hours incubation, it was necessary to dilute the organisms, which grew abundantly, considerably more than those which grew slowly. Two drops of the 18-24 hour broth cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi-murium*, *Proteus*, and *Staphylococcus aureus* were diluted in 10 ml. of sterile saline. The test media were inoculated with 1 drop of the diluted saline suspensions delivered from a 1 ml. pipette. One
drop of the saline suspensions contained between 2,000 to 10,000 organisms (Packer, 1943). The inoculated cultures were incubated at 37°C. Observations were made at 24, and 48 hours, and final results recorded after 72 hours incubation.

It was observed that the sodium azide-containing Edwards medium inhibited completely the various gram-negative organisms without affecting the growth of the streptococci. The original Edwards medium did not prove inhibitory for gram-negative organisms, particularly Pseudomonas aeruginosa. The inulin-containing modified Edwards medium showed red colonies of inulin fermenting streptococci clearly, thus differentiating Str. uberis from Str. agalactiae and Str. dysgalactiae. In the light of the above findings, the following modification of Edwards medium was used in this study:

<table>
<thead>
<tr>
<th>Edwards medium (1933) (modified)</th>
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<tbody>
<tr>
<td>Beef extract</td>
<td>1 liter</td>
</tr>
<tr>
<td>Crystal violet 0.1%</td>
<td>2 ml.</td>
</tr>
<tr>
<td>Defibrinated ox blood</td>
<td>50 ml.</td>
</tr>
<tr>
<td>Inulin</td>
<td>1 gm.</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.5 gm.</td>
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<tr>
<td>pH</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Tryptose broth</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3 gms.</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 gms.</td>
</tr>
<tr>
<td>Tryptose</td>
<td>10 gms.</td>
</tr>
<tr>
<td>Proteose-peptone No. 3</td>
<td>10 gms.</td>
</tr>
</tbody>
</table>
Yeast extract 5 gms.
Water add to 1000 ml.

**Camp test medium** (Christie et al. 1944, modified by Wilson and Slavin, 1950).

5% ox blood agar to which 0.1% esculin was added.

**Isolation procedure**

Swabs taken from milk samples and those collected from various skin sites of the cows were incubated in tryptose broth, pH 6.8, for 24 hours. The broth cultures were then inoculated on sodium azide - Edwards medium (modified) and the plates were incubated at 37°C. for 48 hours. The use of the above medium was found to inhibit all organisms except streptococci. Inulin-fermenting colonies were picked up from the plates by means of a platinum wire and were inoculated into tryptose broth tubes. The tubes were incubated for 18-24 hours. The following media were inoculated with the broth culture to establish biochemical identity of **Str. uberis**:

- Salicin
- Mannite
- Inulin
- Raffinose
- Sorbitol
- Sodium hippurate

The cultures which produced acid in salicin, mannite, sorbitol and inulin but failed to ferment raffinose, and hydrolyzed sodium hippurate were classified as **Str. uberis**.
Sodium hippurate test  The reagent required for the test consisted of 12 gms. of ferric chloride (Fe Cl₃·6H₂O) dissolved in 100 ml. of 2 per cent hydrochloric acid in water.

Before carrying out the test, it was necessary to determine the smallest amount of the above reagent that was required to redissolve the precipitate formed when the reagent was added to 1 ml. of the sterile sodium hippurate medium that had been incubated with the tests.

To one ml. of each streptococcal culture, 0.3 ml. (determined as stated above) of ferric chloride reagent was added. The tube was shaken and was allowed to stand for a few minutes. Culture showing a distinct persistent precipitate was classified as positive. A culture that showed redissolving of the precipitate resulting in a clear solution was taken as negative.

Serological identification

Preparation of extract for Lancefield grouping (Rantz and Randall, 1955)  The organism from each culture was grown in 40 ml. of Todd-Hewitt broth at 37°C, for 24 hours. The culture was centrifuged and the clear supernatant fluid was discarded. Normal saline, 0.5 ml., was added to the sediment to suspend the cells. The cell suspension was autoclaved at 121°C. for 15 minutes at 15 lbs. pressure. It was again centrifuged and the supernatant constituted the streptococcal extract for precipitin test.
Capillary precipitin test  
(1) The sterile end of a 1.5 mm. capillary pipette was dipped into the specific Lancefield group antiserum^1 to allow a column of 2-3 cm. of the serum to rise into the tube.

(2) The excess serum was wiped off from the outside of the tube with a paper tissue.

(3) The tube was inserted into the prepared antigen extract to allow an equal volume (2-3 cm.) to enter the tube.

(4) About 1 cm. air space was allowed to form at both ends.

(5) The excess fluid was wiped off the tube with paper tissue. The pipette was inverted and it was inserted into a plasticine block.

(6) Normal serum controls and streptococcal antigen controls were included in the test.

A positive reaction was indicated by the formation of a fine precipitate in 10-15 minutes.

Characterization

Strains

One hundred and sixty-eight strains of Str. uberis were included in the study for the characterization of the organism. Of these, 151 strains had been isolated during the course of this investigation from 24 experimental cows of the Dairy Farm, Iowa State University. Seventeen strains were kindly supplied by Dr. G. E. Morse, School of Veterinary Medicine, University of Pennsylvania, New Bolton Centre, Pa. 19348.

^1Difco Laboratories, Detroit, Michigan.
Media and procedures

Sugar media

(1) Xylose
(2) Arabinose
(3) Glucose
(4) Fructose
(5) Maltose
(6) Lactose
(7) Sucrose
(8) Trehalose
(9) Melibiose
(10) Inulin
(11) Esculin
(12) Dulcitol

The sugar base medium consisted of:

Beef extract 3 gms.
Yeast extract 3 gms.
Tryptose 15 gms.
Sodium chloride 5 gms.
Andrade's indicator 10 ml.
Water added to 1000 ml.

The fermentable substance was added in the proportion of 0.5 per cent except for esculin which was incorporated at 1 per cent level.
Tryptose broth, pH 9.6

To prepare tryptose broth pH 9.6, the pH of the broth was set at 9.8 with NaOH with a view to obtain the desired pH of 9.6 after autoclaving.

Litmus milk

Bacto-Litmus 0.15 gm.
Skim-milk powder 30.00 gms.
Distilled water 300.00 ml.

The constituents were mixed and allowed to stand in the refrigerator overnight. It was then dispensed, and autoclaved for exactly 8 minutes at 121°C.

Milk containing 0.01 per cent methylene blue
Milk containing 0.1 per cent methylene blue

Gelatin

Tryptose broth 1000 ml.
Gelatin 150 gms.

The gelatin was added to tryptose broth and was melted in the autoclave for 10 minutes. It was dispensed in 3 ml. quantity per tube.

Starch agar

Difco starch agar was used. The ingredients per liter were:

Bacto-Beef extract 3 gms.
Soluble starch, Difco 10 gms.
Bacto-agar 12 gms.
Water added to 1000 ml.
Twenty five gms. of Difco Bacto starch agar was suspended in 1000 ml. cold distilled water and it was heated to boiling to dissolve the medium completely. The medium was autoclaved at 121°C, for 15 minutes.

**Starch hydrolysis**  The test organism was streaked across the center line on the starch agar plate. The plate was incubated for 24-48 hours to obtain good growth. The test was made by flooding the surface of the petri dish with Lugol's iodine. The breadth of the clear zone outside the area of growth indicated the extent of starch destruction. The presence of bluish-purple color, characteristic of the starch-iodine complex, indicated lack of hydrolysis.

**Tyrosine decarboxylase activity**  A differential pH method evolved by Gale\(^1\) (cited by Sharpe, 1948) was used to test the *Str. uberis* cultures for tyrosine decarboxylase activity. This is based on the observation that when tyrosine is decarboxylated in an acid medium, the CO\(_2\) is driven off and there is a change in pH towards the alkaline following the production of the amine.

The basic medium consisted of:

- Peptone 1.0 per cent
- Marmite (yeast extract) 0.1 per cent
- Glucose in glass distilled water 0.2 per cent

The medium was autoclaved at 15 pounds for 20 minutes, filtered, and adjusted to pH 7.3. To half of the medium 0.5 per cent tyrosine was added.

\(^1\)Gale, E. F. 1948. Private communication; cited by Sharpe, M.E. 1948.
The second half did not contain any tyrosine. The two media were filled in 5 ml. volumes in screw capped test tubes, and autoclaved at 10 pounds for 15 minutes.

One test tube of each medium was inoculated with 2 drops (0.06 ml) of a culture of the test organism, grown for 20 hours in tryptose broth, incubated at 37°C. overnight, and the difference in pH between the two media determined by pH meter. The quantity of glucose in the basic medium was such that it was just exhausted when the pH had fallen to the limit of acid tolerance-about 4.0-4.5. If the organism produced tyrosine decarboxylase, the formation in the tyrosine medium of tyramine caused a rise in pH. The difference in pH units between the inoculated basic medium and the inoculated basic medium plus tyrosine was taken as a measure of tyrosine decarboxylase activity. Sharpe (1948) mentioned a difference of 1.4-1.6 pH units to be indicative of high tyrosine decarboxylase activity and a difference of 0.0-0.2 unit as indicative of little or no tyrosine decarboxylase activity.

Growth of *Str. uberis* at 10°C. and 45°C. The broth cultures were incubated for 72 hours in Fisher incubator set at 10°C., and 45°C. respectively.

Survival at 60°C. for 30 minutes A thermostatically controlled water bath was used to study the survival of the test cultures. The water bath was set at 60°C. The broth culture tubes were well immersed in the heated water. A control tube of broth was used to determine the time the cultures took to reach the 60°C. temperature. They were exposed to 60°C. for
30 minutes. The tubes were then incubated at 37°C. for 72 hours, the results were read every 24 hours.

Hydrolysis of arginine

Arginine broth (Niven et al., 1942)

Yeast extract (Difco) 0.5 gm.
Tryptone (Difco) 0.5 gm.
K₂HPO₄ 0.2 gm.
Glucose 0.05 gm.
d-Arginine 0.3 gm.
Water 100 ml.

Nessler reagent

Solution A. Anhydrous 10 gms.
Mercuric chloride
Anhydrous potassium iodide 7 gms.
Ammonia-free water 20 ml.

Solution B. Sodium hydroxide 16 gms.
Ammonia-free water 15 ml.

Solution A was added slowly, with stirring, to cold solution B. The combined mixture was diluted to 100 ml. with ammonia-free water.

A tube of arginine broth was inoculated with 0.1 ml. of a 24 hour-old tryptose broth culture of the organism. It was incubated for 48 hours. One loopful of the culture was placed in a depression of a white spot plate, and one loopful of Nessler reagent was added to it, and mixed.
A positive reaction was indicated by the development of an orange color. If the culture was negative, the mixture remained colorless or was pale yellow in color.

**Tolerance test in bile salts (Kenner et al., 1960)**

**Brain heart infusion broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion from calf brain</td>
<td>200 gms.</td>
</tr>
<tr>
<td>Infusion from beef heart</td>
<td>250 gms.</td>
</tr>
<tr>
<td>Proteose peptone, Difco</td>
<td>10 gms.</td>
</tr>
<tr>
<td>Bacto-Dextrose</td>
<td>2.0 gms.</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 gms.</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>2.5 gms.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml.</td>
</tr>
</tbody>
</table>

**10 per cent bile salt broth**

10 ml. of sterile 10 per cent oxgall solution was added to 90 ml. of sterile brain heart infusion broth.

**20 per cent bile salt broth**

20 ml. of sterile 10 per cent oxgall solution was added to 80 ml. of sterile brain heart infusion broth.

**40 per cent bile salt broth**

40 ml. of sterile 10 per cent oxgall solution was added to 60 ml. of sterile brain heart infusion broth.
The media tubes were each inoculated with 3 drops of a 24 hour tryptose broth culture of the organism to be tested. They were incubated at 37°C. for 3 days and then examined for growth.

**Bacteriophages**

A total of 147 strains of *Str. uberis* were tested for phage production and phage sensitivity. These strains had been isolated from 12 body sites and milk samples of 24 cows of Iowa State University dairy farm during a survey carried out during 1967-68.

Eleven cultures of enterococci (Lancefield group D) and 17 cultures of streptococci belonging to groups A, B, C, E, and L were also examined for sensitivity to four *Str. uberis* phages, isolated in this study. The strains of enterococci were obtained from Dr. P. A. Hartman, Department of Bacteriology, and cultures of groups A, B, C, E, and L were kindly supplied by Dr. R. A. Packer, Head, Department of Veterinary Microbiology, Iowa State University.

**Culture media**

**Tryptose broth**

- Beef extract: 3 gms.
- Sodium chloride: 5 gms.
- Tryptose: 10 gms.
- Proteose peptone No. 3: 10 gms.
- Yeast extract: 5 gms.
- Water add to: 1000 ml.
- pH 7.4
Tryptose - serum agar

Tryptose broth

Agar 1.5 per cent
Normal horse serum 10 per cent

Soft agar

Agar 0.7 gm.
Peptone 1.0 gm.
Water 100 ml.
pH 7.0

Isolation of phages

Cultures of *Str. uberis* were examined for their phage producing activity. Ten ml. of tryptose broth was inoculated with 0.1 ml. of a 24 hour broth culture of the organism. It was incubated for 24 hours. The culture was centrifuged at 2000 r.p.m. for 20 minutes. Five ml. of the supernatant fluid was pipetted into a sterile tube. One drop of chloroform from a 1 ml. standard pipette was added to the supernatant fluid and it was shaken vigorously a few times to saturate it with chloroform. The mixture was left at room temperature for 10 minutes to permit chloroform to act. It was then centrifuged for 20 minutes at 2000 r.p.m. One ml. of the supernatant was collected by means of a Pasteur pipette and stored in the refrigerator. Alternatively in some cases, the supernatant obtained after first centrifugation was filtered through a 220 μm millipore filter, instead of chloroform treatment.
The cultures were examined for evidence of phage activity in groups of nine by testing each of them against the filtrates obtained from the cultures. This was done by flooding a plate of tryptose-serum agar with 1 ml. of a 24 hour broth culture of the organism. The back of the plate was marked so as to divide it into nine areas. One drop of each of the 9 supernatants or filtrates was added to the respective areas. The drops on the plate were allowed to dry for about 15-20 minutes and then the plates were incubated overnight at 37°C. with the lid on the upper side. The plates were examined next morning for the presence or absence of lysis.

Some cultures proved lysogenic. The lysogenic strain was induced by exposure to ultraviolet light. A 24 hour broth culture of the organism was centrifuged and the bacteria suspended in an equal volume of sterile 0.85 per cent sodium chloride solution. A shallow layer, 2 mm. in depth, of the suspension was exposed for 10 seconds to 2550 erg/cm² sec. as determined by Black-Ray ultraviolet intensity meter.¹ The suspension was then diluted 1 to 10 in broth and incubated at 37°C. for 3 hours. The culture was then cooled, centrifuged, and the supernatant titered for plaque-forming units.

Propagation and assay of phage

The agar-overlay method of Gratia (1936), as described by Adams (1959), was employed.

Two and a half milliliters of 0.7 per cent soft agar, contained in a tube, was melted in a boiling water bath and cooled in a 46°C. water bath.

¹Ultraviolet Products, Inc., San Gabriel, California.
Two drops of an actively growing broth culture of *Str. uberis* strain and 0.1 ml. of the phage were pipetted into the tube of melted agar. The mixture was poured immediately over the surface of an agar plate containing 20 ml. of 1.5 per cent tryptose-serum agar. Both the 1.5 per cent serum agar and the soft agar were allowed to harden with the petri dish resting on a leveled sheet of glass. The plate was incubated over night. Next morning it was sharp frozen at -80°F. for 20 minutes. The contents of the plate were then allowed to thaw in a slanting position. The resulting fluid was collected in a test tube and centrifuged. It was filtered through a 220 μm millipore filter or treated with chloroform as described above. The routine test dilution was determined by assaying the filtrate by the agar-overlay method. Plates were examined for plaques or areas of confluent lysis after 8, 12, 18 and 24 hours of incubation at 37°C.

**Examination of cultures for phage sensitivity**

The phage sensitivity of each strain was determined by flooding a tryptose-serum agar plate with 1 ml. of a 24 hour broth culture of the organism. The plate was rocked to and fro gently to inoculate it evenly with the test culture. The plate was allowed to dry at room temperature for about one hour. The back of the plate was marked so as to divide it into 12 areas. One drop of the routine test dilution of each of the 11 phages was added to the respective areas marked numerically. One drop of tryptose broth was added to the 12th area to serve as a control for reading the results. The drops on the plate were allowed to dry for about 15-20 minutes and then the plates were incubated over night at 37°C. They were examined next morning for evidence of lysis.
RESULTS

Characterization

One hundred and sixty-eight strains of *Str. uberis* were subjected to 32 tests, such as, fermentation of carbohydrates, hydrolysis of sodium hippurate, esculin, arginine, and starch, liquefaction of gelatin, growth at 10°, 45°, and 60°C., growth in 6.5 per cent and 4.0 per cent sodium chloride, litmus milk, milk containing 0.01 per cent, and 0.1 per cent methylene blue, and broth pH 9.6, tolerance tests in 10, 20, and 40 per cent bile salts, tyrosine decarboxylase test, and growth on blood agar for evidence of hemolysis.

Of the 168 strains of *Str. uberis*, all fermented salicin, mannitol, inulin, trehalose, glucose, maltose, fructose, sucrose, and sorbitol. Lactose was fermented by all except two strains. Twenty-one (12.5 per cent) strains showed weak positive reaction in sorbitol, 11 (6.5 per cent) weak reaction in inulin, and 11 (6.5 per cent) were weakly positive in mannitol. All the strains hydrolyzed sodium hippurate. Eight (4.8 per cent) of the 168 strains, however, showed weak positive reaction in sodium hippurate. Esculin and arginine were hydrolyzed by all the strains. None of the strains hydrolyzed starch.

One hundred and thirty-eight (82.1 per cent) of the 168 strains grew at 10°C., 161 (95.8 per cent) grew at 45°C., and 127 (75.6 per cent) were able to survive a temperature of 60°C. for 30 minutes. One hundred and fifty-one (89.9 per cent) strains showed growth in 4.0 per cent sodium chloride tryptose broth, and 28 (16.6 per cent) were able to grow even in
the presence of 6.5 per cent sodium chloride. Most of the strains showed acid production, coagulation, and reduction in litmus milk: 165 (98.2 per cent) produced acid, 158 (94.0 per cent) produced clot, and 163 (97.0 per cent) caused reduction. There was no strain which was completely negative to litmus milk. The reaction in litmus milk appeared to be influenced by the dose of inoculum. With three drops (0.09 ml.) from a Pasteur pipette, more strains produced clotting and reduction than when the litmus milk was inoculated by means of a platinum loop.

One hundred and fifty-nine (94.6 per cent) out of 168 strains of Streptococcus uberis did not grow in milk containing 0.1 per cent methylene blue. One hundred and fifty-three (91.1 per cent) strains grew in milk containing 0.01 per cent methylene blue. Fifteen (8.9 per cent) strains did not grow even in 1:10,000 methylene blue milk.

Observations recorded on tolerance tests using 10, 20, and 40 per cent bile salts revealed that 142 (84.5 per cent) strains grew in 10 per cent, 102 (60.7 per cent) showed growth in the presence of 20 per cent, and 37 (22.0 per cent) of the strains were able to grow even in the presence of 40 per cent bile salts. Strains which failed to grow in 10 per cent also did not grow in higher concentrations of bile salts.

Tyrosine decarboxylase activity was tested on 168 strains of Streptococcus uberis, using a differential pH method employed by Sharpe (1948). Of the 168 strains, 95 (56.5 per cent) showed no tyrosine decarboxylase activity, 20 (11.9 per cent) caused a pH rise of less than 0.1 unit, 45 (26.8 per cent) showed a rise of 0.1 to 0.2 pH unit, and 8 (4.8 per cent) strains caused a rise of 0.2 to 0.3 pH unit.
The above observations indicated that 160 out of 168 strains of *Strep. uberis* showed little or no tyrosine decarboxylase activity. Eight strains (4.8 per cent) showed a rise of 0.2 to 0.3 (average 0.28) pH unit which can not be regarded as indicative of any significant tyrosine decarboxylase activity. Consequently all the strains are shown in Table 2 to be negative for tyrosine decarboxylase activity.

The hemolytic activity of the strains was tested by inoculating them on 5 per cent ox blood agar plates. The plates were incubated at 37°C and examined at the end of 24 and 48 hours incubation. Of the 168 strains of *Strep. uberis*, 152 (90.5 per cent) were found to be non-hemolytic, 12 (7.1 per cent) alpha-hemolytic, and 4 (2.4 per cent) showed narrow-beta type of hemolysis.

The following table gives a summary of the biochemical and physiological characteristics of 168 strains of *Strep. uberis*, examined in this study.

**Table 2. Summary of biochemical and physiological characteristics of Streptococcus uberis**

<table>
<thead>
<tr>
<th>So. no.</th>
<th>Test</th>
<th>No. cultures tested</th>
<th>No. tests positive</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Xylose</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Arabinose</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Glucose</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Fructose</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Maltose</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Lactose</td>
<td>168</td>
<td>166</td>
<td>98.8</td>
</tr>
<tr>
<td>7</td>
<td>Sucrose</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Trehalose</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Melibiose</td>
<td>168</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10</td>
<td>Raffinose</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>Inulin</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>Mannitol</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2. (Continued)

<table>
<thead>
<tr>
<th>So. no.</th>
<th>Test</th>
<th>No. cultures tested</th>
<th>No. tests positive</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Sorbitol</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>Dulcite</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>Salicin</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>Esculin</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>Gelatin</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>Hydrolysis of sodium hippurate</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>Hydrolysis or arginine</td>
<td>168</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>Growth at 10°C.</td>
<td>168</td>
<td>138</td>
<td>82.1</td>
</tr>
<tr>
<td>21</td>
<td>Growth at 45°C.</td>
<td>168</td>
<td>161</td>
<td>95.8</td>
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<tr>
<td>22</td>
<td>Survival at 60°C. for 30 minutes</td>
<td>168</td>
<td>127</td>
<td>75.6</td>
</tr>
<tr>
<td>23</td>
<td>Growth in 6.5% NaCl</td>
<td>168</td>
<td>28</td>
<td>16.6</td>
</tr>
<tr>
<td>24</td>
<td>Growth in 4.0% NaCl</td>
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<td>151</td>
<td>89.9</td>
</tr>
<tr>
<td>25</td>
<td>Growth in litmus milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reddening</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Coagulation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Reduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Growth in milk containing 0.1% methylene blue</td>
<td>168</td>
<td>9</td>
<td>5.4</td>
</tr>
<tr>
<td>27</td>
<td>Growth in milk containing 0.01% methylene blue</td>
<td>168</td>
<td>153</td>
<td>91.1</td>
</tr>
<tr>
<td>28</td>
<td>Growth in broth, pH 9.6</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>Hydrolysis of starch</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>Tyrosine decarboxylation</td>
<td>168</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>Tolerance of bile salts</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>concentration: 10%</td>
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</tr>
<tr>
<td></td>
<td>20%</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>40%</td>
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</tr>
<tr>
<td>32</td>
<td>Reaction on blood agar</td>
<td>168</td>
<td>152</td>
<td>90.5</td>
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<tr>
<td></td>
<td>non-hemolytic</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
<td></td>
<td>7.1</td>
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<td>alpha-hemolytic</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>narrow beta-hemolytic</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Thirty-one (20.5 per cent) strains out of 151 isolated, reacted with Lancefield group E antiserum.

Of the 151 strains of *Streptococcus uberis*, isolated in this study, 17 (11.25 per cent) proved positive to the CAMP test. Figure 1 shows CAMP reaction exhibited by six of the positive strains of *Streptococcus uberis*.

There were 19 strains which resembled *Streptococcus uberis* in some characters but were inulin-negative. On a closer study, it was observed that each one of them, in addition to being inulin-negative, also showed some other reactions which were not typical of *Streptococcus uberis* species. Table 3 shows the variations observed in them.

None of the strains (Table 3) reacted with Lancefield group E antiserum.

Besides, *Streptococcus uberis*, other organisms isolated from skin sites and milk samples included *Streptococcus bovis* (237), *Streptococcus zooepidemicus* (65), *Streptococcus faecalis* (35), *Streptococcus equisimilis* (25), and *Streptococcus dysgalactiae* (2).

**Bacteriophage Sensitivity**

A battery of 11 phages of *Streptococcus uberis* was selected to test the sensitivity of 147 strains of *Streptococcus uberis*, isolated from the body skin and milk samples of the experimental cows. Table 4 shows the number of strains sensitive to the different phages.

\[1\text{The figures in brackets are the number of strains isolated.}\]
Figure 1. CAMP reaction exhibited by *Str. uberis* strains
Table 3. Variations in biochemical and physiological characteristics of inulin-negative streptococci in relation to *Str. uberis* species

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Ara-binose</th>
<th>Melibiose</th>
<th>Sucrose</th>
<th>Trehalose</th>
<th>Growth in 6.5% NaCl broth</th>
<th>Gela-tin liquefaction</th>
<th>Tyrosine decarboxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pH 9.6</td>
<td>methylene blue milk</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>44</td>
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<tr>
<td>46</td>
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<td>+</td>
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<td>54</td>
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<td>132</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>174</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>186</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>187</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>188</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>189</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Typi-cal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Str. uberis</em></td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4. Phage-sensitivity of 147 strains of *Str. uberis*

<table>
<thead>
<tr>
<th>Phage</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
<th>XI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of <em>Str. uberis</em> strains found sensitive</td>
<td>29</td>
<td>91</td>
<td>5</td>
<td>118</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>9</td>
<td>11</td>
<td>30</td>
</tr>
</tbody>
</table>

Variation was noticed in the degree of lysis caused by the different phages. The following table gives the number of strains of *Str. uberis* showing variation in the degree of lysis.

Table 5. Variation in the degree of lysis shown by strains of *Str. uberis*

<table>
<thead>
<tr>
<th>Phage</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
<th>XI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confluent lysis</td>
<td>13</td>
<td>11</td>
<td>3</td>
<td>57</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Partial lysis</td>
<td>16</td>
<td>80</td>
<td>2</td>
<td>61</td>
<td>11</td>
<td>7</td>
<td>7</td>
<td>14</td>
<td>7</td>
<td>11</td>
<td>18</td>
</tr>
</tbody>
</table>

The above results indicated that of the 11 phages used for testing the sensitivity of 147 strains of *Str. uberis*, four phages, No. I, II, IV and XI, were more active than the remaining seven phages. Phage IV lysed 118 strains, phage II caused lysis of 91 strains, phage XI lysed 30, and phage I produced lysis of 29 strains. Figure 2 shows areas of confluent lysis produced by the four phages.

Nineteen strains out of 147 tested were not sensitive to any of the 11 phages. Of the 128 sensitive strains, 126 were lysed by one or more of
Figure 2. Bacteriophage activity of *Str. uberis* phages
the four phages No. I, II, IV and XI. The two strains resistant to all
the four phages were No. 65 and 94. Strain No. 65 was, however, sensitive
to phage X, and strain No. 94 was lysed by phage VIII.

All the 91 strains except strain No. 66, that were lysed by phage
II, were also sensitive to phage IV. Of the 118 strains lysed by phage IV,
91 (77.1 per cent) were also lysed by phage II. These host-range studies
seemed to indicate that phages II and IV were fairly closely related to
each other.

Of the 29 strains lysed by phage I, 22 of them were sensitive to
phage II, 25 to phage IV, and 9 to phage XI. Of the 30 strains sensitive
to phage XI, 18 were also lysed by phage II, and 27 were sensitive to phage
IV.

An analysis of the lytic ability of the four phages revealed that of
the 126 strains, sensitive to one or more of these phages, all except four
were sensitive to phage IV. This indicated that there was considerable
uniformity in the strains of Str. uberis studied. This is in conformity
with the biochemical reactions of the strains of the organism studied. It
may, however, be mentioned that five inulin-negative strains, tested in this
study, were also sensitive to one or more of these phages.

Eleven cultures of enterococci were tested with the four Str. uberis
phages. None of them showed any lysis. Five cultures of Str. agalactiae,
one of Str. dysgalactiae, three of Str. pyogenes, two of Str. canis, one of
Str. zooepidemicus, one of Str. equisimilis, one of Str. equi, two of Str.
suis, and one of streptococcus group L were also examined. One strain of
Str. pyogenes showed cross-reaction with phage II, and one strain of Str. canis showed partial lysis with phages II and XI.

Lysogenic strains

Of the 79 cultures of Str. uberis examined for lysogeny, 23 (29.1 per cent) were found to be lysogenic.

The work reported above is only a preliminary study of the bacteriophage activity of Str. uberis. More work needs to be done to explore the possibility of using it for the identification of Str. uberis.

Incidence and Ecology

A total of 6836 swabs from 12 skin sites of the body and 568 milk samples from 24 experimental cows were examined for Str. uberis infection during the period from October, 1967 to October, 1968. One hundred and seventy-two strains of Str. uberis were isolated. Of the 1704 swabs examined from udder and teat surfaces, 103 (6.0 per cent) yielded Str. uberis. Fifty-one (1.0 per cent) swabs out of 5132 collected from nine other sites on the body of cows, mentioned under 'Materials and Methods', proved positive for Str. uberis infection. The examination of 568 milk samples revealed 18 (3.2 per cent) of them to be positive for Str. uberis. The following table shows the distribution of strains, isolated from the various sites and from milk samples, grouped under three categories.

The percentage isolation rate was adjusted for the udder surface. Two udder surface swabs were taken from each cow but as the udder was considered as a single site, the total isolates from the udder surfaces,
Table 6. Broad distribution of isolates of *Str. uberis* from skin sites and milk samples

<table>
<thead>
<tr>
<th>Season</th>
<th>Udder and teat surfaces</th>
<th>Nine other sites of the body</th>
<th>Milk samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total samples</td>
<td>Total samples</td>
<td>Total samples</td>
</tr>
<tr>
<td>Fall</td>
<td>462</td>
<td>1386</td>
<td>154</td>
</tr>
<tr>
<td>Winter</td>
<td>360</td>
<td>1080</td>
<td>120</td>
</tr>
<tr>
<td>Spring</td>
<td>423</td>
<td>1269</td>
<td>141</td>
</tr>
<tr>
<td>Summer</td>
<td>459</td>
<td>1397</td>
<td>153</td>
</tr>
<tr>
<td>Total</td>
<td>1704</td>
<td>5132</td>
<td>568</td>
</tr>
<tr>
<td>Per cent positive</td>
<td>6.0</td>
<td>1.0</td>
<td>3.2</td>
</tr>
</tbody>
</table>

numbering 70, were reduced to 35. Observations recorded showed that the udder skin accounted for the greatest percentage of isolates, 25.55, followed by teat surfaces (24.09), lips, and belly, each 13.87, side of the chest (3.65), sacrum (2.19), nostrils (1.46), posterior part of vagina, posterior part of rectum, and caudal folds, each 0.73, and milk accounted for 13.14 per cent of the isolates. Table 7 gives the distribution of total isolates in the different sites.

A histogram showing the distribution of isolates, as shown in Table 7, was constructed. It is presented in Figure 3.

The isolates were not confined to a few cows but were distributed throughout the entire herd. Table 8 shows the distribution of the isolates amongst the cows.
Table 7. Distribution of total isolates of *Str. uberis* in the different sites

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of isolates</th>
<th>No. corrected</th>
<th>% corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Udder surfaces</td>
<td>70</td>
<td>35</td>
<td>25.55</td>
</tr>
<tr>
<td>Teat surfaces</td>
<td>33</td>
<td>33</td>
<td>24.09</td>
</tr>
<tr>
<td>Lips</td>
<td>19</td>
<td>19</td>
<td>13.87</td>
</tr>
<tr>
<td>Belly</td>
<td>19</td>
<td>19</td>
<td>13.37</td>
</tr>
<tr>
<td>Side of chest</td>
<td>5</td>
<td>5</td>
<td>3.65</td>
</tr>
<tr>
<td>Sacrum</td>
<td>3</td>
<td>3</td>
<td>2.19</td>
</tr>
<tr>
<td>Nostrils</td>
<td>2</td>
<td>2</td>
<td>1.46</td>
</tr>
<tr>
<td>Posterior part of vagina</td>
<td>1</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td>Posterior part of rectum</td>
<td>1</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td>Caudal folds</td>
<td>1</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td>Milk</td>
<td>18</td>
<td>18</td>
<td>13.14</td>
</tr>
</tbody>
</table>

Month-wise distribution of isolates

The investigation was conducted for one full year. The data collected on the isolation of strains of *Str. uberis* from different skin sites and milk samples of cows was analyzed to study the month-wise distribution of isolates. The skin swabs and milk samples from the two groups of cows, each consisting of 12 animals, were collected four times every month except for February and December, when they were collected three times during the month. The number of strains isolated per 24 cows during each month was calculated by dividing the number of strains obtained during the month
Figure 3. Histogram showing distribution of isolates of *Str. uberis* in the different sites.
DISTRIBUTION OF ISOLATES OF *STREPTOCOCCUS UBERIS*

**LEGEND:**

- **A** UDDER SURFACES
- **B** TEAT SURFACES
- **C** LIPS
- **D** BELLY
- **E** MILK
- **F** CHEST
- **G** SACRUM
- **H** NOSTRILS
- **I** POSTERIOR PART OF VAGINA
- **J** POSTERIOR PART OF RECTUM
- **K** CAUDAL FOLDS
Table 8. Distribution of isolates amongst cows of the experimental herd

<table>
<thead>
<tr>
<th>Cow no.</th>
<th>Total isolates per cow</th>
<th>Cow no.</th>
<th>Total isolates per cow</th>
</tr>
</thead>
<tbody>
<tr>
<td>5613</td>
<td>14</td>
<td>4439</td>
<td>6</td>
</tr>
<tr>
<td>5586</td>
<td>14</td>
<td>5408</td>
<td>6</td>
</tr>
<tr>
<td>5063</td>
<td>12</td>
<td>5247</td>
<td>5</td>
</tr>
<tr>
<td>5574</td>
<td>11</td>
<td>5518</td>
<td>5</td>
</tr>
<tr>
<td>5375</td>
<td>10</td>
<td>5556</td>
<td>5</td>
</tr>
<tr>
<td>5539</td>
<td>10</td>
<td>5429</td>
<td>5</td>
</tr>
<tr>
<td>5600</td>
<td>10</td>
<td>5301</td>
<td>4</td>
</tr>
<tr>
<td>5116</td>
<td>9</td>
<td>5146</td>
<td>4</td>
</tr>
<tr>
<td>5413</td>
<td>9</td>
<td>4647</td>
<td>3</td>
</tr>
<tr>
<td>5420</td>
<td>8</td>
<td>5393</td>
<td>3</td>
</tr>
<tr>
<td>5290</td>
<td>7</td>
<td>5253</td>
<td>3</td>
</tr>
<tr>
<td>5316</td>
<td>7</td>
<td>5388</td>
<td>2</td>
</tr>
</tbody>
</table>

by 4/2 or 3/2 depending upon the number of times the swabs and milk samples were collected during the month. Results so obtained indicated that the incidence of Str. uberis infection was highest in the month of December, 16.67 strains per 24 cows, followed by January, February, November, April, September and October, March, May and August, July, and the least in June, the respective figures being 16.50, 11.33, 10.00, 7.50, 7.00, 7.00, 6.00, 3.50, 3.50, 3.00 and 1.00. These results are summarized in Table 9.
Table 9. Month-wise distribution of isolates of **Str. uberis** from skin sites and milk samples of cows

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of isolates month-wise</td>
<td>33</td>
<td>17</td>
<td>12</td>
<td>15</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>14</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>No. times sample collections were made each month</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of isolates per 2* cows</td>
<td>16.50</td>
<td>11.33</td>
<td>6.00</td>
<td>7.50</td>
<td>3.50</td>
<td>1.00</td>
<td>3.00</td>
<td>3.50</td>
<td>7.00</td>
<td>7.00</td>
<td>10.00</td>
<td>16.67</td>
</tr>
</tbody>
</table>

A histogram was constructed to show month-wise distribution of isolates of **Str. uberis** from skin sites and milk samples of cows. It is presented in Figure 4.

**Seasonal distribution of isolates**

The above results when interpreted in relation to the seasons indicated that the incidence of **Str. uberis** infection was highest in winter (December to February), followed by fall (September to November), spring (March to May), and the least in summer (June to August), the respective figures being 44.50, 24.00, 17.00, and 7.50. A histogram showing seasonal distribution of **Str. uberis** isolates is presented in Figure 5.

**Relation of Str. uberis infection to age**

Of the 11 cows showing mammary gland infection with **Str. uberis**, three were in the 3-4 year age-group, two in the 4-5 year age-group, one in the 5-6 year group, two in the 6-7 year, one in the 7-8 year, one in the 9-10 year, and one in the 11-12 year age-group. These observations
Figure 4. Histogram showing month-wise distribution of isolates of *Str. uberis*
MONTH-WISE DISTRIBUTION OF ISOLATES OF STR. UBERIS
Figure 5. Histogram showing seasonal distribution of isolates of *Str. uberis*
SEASONAL DISTRIBUTION OF ISOLATES OF \textit{STR. UBERIS}
indicated that the incidence of *Str. uberis* infection of the mammary gland increased as the cows advanced in age as five cows out of six (83.33 per cent) in the age-groups from 6 to 12 years were found infected as against six out of 18 (33.33 per cent) cows in the age-groups from 3 to 6 years.

**Relation of *Str. uberis* infection to the stage of lactation**

The analysis of 172 strains of *Str. uberis* isolated during the study revealed that the maximum number of strains, 124, were isolated during mid and late lactation (8th week to drying off), followed by 35 during early lactation (from calving to 7th week), and the least, 13, during the dry period. The mid and late lactation period comprised of nearly 36 weeks, whereas the early lactation period consisted of seven weeks. The data was therefore corrected to calculate the number of isolates per week to obtain a uniform basis for comparative study. The data, so corrected, indicated that the incidence of *Str. uberis* infection was the highest during the early lactation period, followed by the mid and late lactation period, and the least during the dry period, the three figures being 5.00, 3.44, and 1.44 respectively. The results are summarized in the following table.

<table>
<thead>
<tr>
<th></th>
<th>Early lactation</th>
<th>Mid and late lactation</th>
<th>Dry period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains</td>
<td>35</td>
<td>124</td>
<td>13</td>
</tr>
<tr>
<td>isolated per week</td>
<td>5.00</td>
<td>3.44</td>
<td>1.44</td>
</tr>
<tr>
<td>isolated per week</td>
<td>5.00</td>
<td>3.44</td>
<td>1.44</td>
</tr>
</tbody>
</table>
DISCUSSION

Characterization

In the diagnosis of bovine mastitis, *Str. uberis* is generally distinguished from *Str. agalactiae* and most other well defined species by the use of relatively few tests: esculin splitting, hydrolysis of sodium hippurate, production of ammonia from arginine, and action on litmus milk. This procedure serves the purpose quite well, especially when the precise origin of the organisms isolated is known. The characterization of *Str. uberis*, however, needs a large number of biochemical and physiological tests in order to define the limits of this species to differentiate it from other undefined non-pathogenic organisms. Several tests have been used by various workers. Typical strains of *Str. uberis* ferment mannitol, salicin, inulin, trehalose, lactose, sorbitol, glucose, fructose, and maltose with production of acid. In this study, of the 168 strains examined, all of them fermented glucose, fructose, maltose, sucrose, trehalose, mannitol, sorbitol and salicin. These results are in conformity with those described by Little et al. (1946); Sceley (1951); Bergey (1957); Stableforth (1959); Merchant and Packer (1967); and Cullen (1967). Cullen (1967), however, reported 32 sorbitol-negative strains of *Str. uberis* out of 865 examined from clinical and subclinical cases of mastitis and from the skin. He added that sorbitol-negative strains, although unusual, must be considered within the species, as they occurred in organisms with otherwise normal patterns, and showed the correct percentage of group E strains among them.
Slot (1958) found seven out of 156 strains of *Str. uberis* which were sorbitol-negative. Heeschen and Meyer (1965) reported 12 sorbitol-negative strains of *Str. uberis* out of 46 tested by them.

Fermentation of inulin is a very important characteristic of *Str. uberis*. Of the 168 strains of *Str. uberis* tested, all of them fermented inulin. This is in agreement with the findings of Minett (1934) who reported inulin-positive reaction as a characteristic of his mastitis streptococci group III (*Str. uberis*) organisms. Ferguson (1938) tested 19 strains of *Str. uberis* isolated from quarter milk samples and found all of them to be inulin-positive. Slot (1958) examined 156 strains of *Str. uberis* and reported that they were all inulin-positive. Pedersen (1960) mentioned that fermentation of inulin within 24 hours was a reliable means of distinguishing *Str. uberis*. Cullen (1967) considered fermentation of inulin as an essential characteristic of *Str. uberis*. However, inulin-negative strains of *Str. uberis* have also been reported by some workers. Slanetz and Naghski (1940) reported that of the 83 cultures of *Str. uberis* tested, nine cultures failed to ferment inulin. Little et al. (1946) mentioned that about 10 per cent of the strains, studied by them, although possessing many of the cultural characteristics of *Str. uberis*, were found negative in inulin. They indicated that such cultures were, however, serologically identical with inulin-positive strains. Seeley (1951) examined 52 *Str. uberis* cultures and found 43 of them inulin-positive and nine inulin-negative. Heeschen and Meyer (1965) tested 46 strains of *Str. uberis* (41 group E-positive and 5 serologically negative) for fermentation of inulin and found 34 of them inulin-positive and 12 gave a negative
reaction in inulin. The inulin-negative strains were encountered in both group E-positive and serologically negative Str. uberis cultures.

It was observed that one strain out of 168 examined required incubation for 96 hours before it proved inulin-positive. Another culture, which had been stored for some months without subculturing, proved positive on incubation for 10 days. These observations indicate the desirability of incubating inulin tubes, which fail to ferment, for at least 10 days before declaring them inulin-negative, especially when the cultures have been stored for some time.

It may be mentioned that in this study there were 19 strains (Table 3) which possessed some biochemical properties characteristic of Str. uberis but were inulin-negative. On a closer study, it was observed that each one of them showed some other characters which were not typical of Str. uberis species, such as growth in 1:1,000 methylene blue milk, growth at pH 9.6, arabinose-positive, lactose-negative, sucrose-negative. None of them was found to react with Lancefield group E antiserum. These strains were, therefore, not included in the Str. uberis species. Similar observations had also been recorded by Little (1940) who mentioned that certain strains of Str. uberis differed from the normal only in their failure to ferment inulin. He reported that 17 inulin-negative strains, studied by him, possessed other cultural characteristics not typical for Str. uberis and he suggested that such strains should be regarded as a-typical udder streptococci. Stableforth (1937) had noticed two similar cultures received from co-workers for serological study. Cullen (1967) also drew attention to inulin-negative strains and suggested that such
strains should not be included in the Str. uberis species as they were nearly always atypical in some other respect as well and were never group E positive.

The ability to ferment mannitol seems to be an especially constant attribute of Str. uberis (Seeley, 1951). The 168 strains of Str. uberis examined in this study proved mannitol-positive. This is in conformity with the results reported by Diernhofer (1932); Minett (1934); Little (1940); Little et al. (1946); Seeley (1951); Bergey (1957); Stableforth (1959); Merchant and Packer (1967); and Cullen (1967). Slot (1958), however, reported that of the 156 strains of Str. uberis, examined by him, seven failed to ferment mannitol. Similarly, Heeschen and Meyer (1965) found four out of 46 strains of Str. uberis, which did not ferment mannitol.

Fermentation of lactose is characteristic of Str. uberis and this reaction differentiates it from group E streptococci of swine origin, which do not generally produce acid in lactose (Merchant and Packer, 1967; Thal and Moberg, 1953; and Brown and Shuman, 1969). Two strains of Str. uberis out of 168, examined in this investigation, were found to be lactose-negative, although they were inulin-positive and possessed other characteristics typical of Str. uberis. Slot (1958) also reported two lactose-negative strains of Str. uberis out of 156 that he studied. It may be mentioned that a majority of the strains examined in this work produced acid in lactose on incubation at 37°C. for 24 hours. Six strains, found negative on incubation for 24 hours, proved positive at the end of 48 hours.

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incubation, and one strain needed incubation for 96 hours before it fermented lactose. These observations indicated the desirability of incubating lactose tubes, inoculated with Str. uberis, for at least 4 days before declaring them lactose-negative.

Diernhofer (1932) characterized Str. uberis species and mentioned that it did not ferment raffinose. None of the 168 strains examined in this investigation produced acid from raffinose. This is in agreement with the findings reported by Slanetz and Naghashi (1940) who examined 83 cultures of Str. uberis; Little et al. (1946); Slavin (1948); Bergey (1957); Stableforth (1959); and Merchant and Packer (1967). Seeley (1951) mentioned that only one out of 52 cultures of Str. uberis gave a positive reaction in raffinose. A few raffinose-positive cultures of Str. uberis were also reported by Slot (1958); Heeschen and Meyer (1965); and Cullen (1967). Cullen (1967) suggested that since the raffinose-positive strains had the same proportion of group E positives as the normal strains, there seemed good evidence for including them in the Str. uberis species. It may be mentioned that of the 43 raffinose-positive cultures examined in this investigation, none reacted with Lancefield group E antiserum.

Hydrolysis of esculin is one of the cardinal characteristics of Str. uberis. It has been used to distinguish it from Str. agalactiae (Wilson and Slavin, 1950). Of the 168 strains of Str. uberis, tested, all of them hydrolyzed esculin. Similar results were reported by Ferguson (1938); Little (1940); Slanetz and Naghski (1940); Seeley (1951); Slot (1958); and Stableforth (1959). Wilson and Slavin (1950) made use of the esculin
splitting property of *Str. uberis* by incorporating 0.1 per cent of esculin in 5 per cent blood agar to differentiate it from *Str. agalactiae*, which does not split esculin. Heeschen and Meyer (1965), however, reported that one out of 46 strains of *Str. uberis* failed to split esculin. It was Lancefield group E positive. Cullen (1967) considered hydrolysis of esculin as an important property of *Str. uberis* and when used in conjunction with sodium hippurate test, the two reactions were found very reliable. He indicated that esculin splitting streptococci found in large numbers in milk taken aseptically from an udder showing obvious inflammation were likely to be *Str. uberis*, although small numbers of other types might be mistakenly labelled as *Str. uberis*. Edwards (1932) was the only worker who reported that his group III strains did not split esculin. This difference seems difficult to understand.

Diernhofer (1932) reported that *Str. uberis* species, as originally characterized by him, split sodium hippurate. Results obtained in this study on 168 cultures of *Str. uberis* showed that all the strains hydrolyzed sodium hippurate, although eight of them were weakly positive. These results supported the findings already reported by Ferguson (1938); Little (1940); Seeley (1951); Stableforth (1959); Pedersen (1960); and Cullen (1967). Seeley (1951) found that about 10 per cent of the 52 strains were weakly positive. He indicated that it appeared that the ability strongly to hydrolyze sodium hippurate was a variable trait of the species. Some sodium hippurate-negative strains of *Str. uberis* were also reported. Slanetz and Naghski (1940) found that 15 out of 83 cultures, examined by them, did not produce hydrolysis of sodium hippurate. Similar findings
were reported by Slot (1958); and Heeschen and Meyer (1965). Cullen (1967) considered hydrolysis of sodium hippurate as an essential test for the identification of \textit{Staph. uberis} as it was very reliable and no group E positive otherwise typical patterns were seen which were negative to either of the sodium hippurate or esculin tests.

The observations recorded in this study revealed that most of the strains showed acid production, coagulation and reduction of litmus milk: 165 (98.2 per cent) of the 168 cultures of \textit{Staph. uberis} produced acid, 158 (94.0 per cent) produced clot, and 163 (97.0 per cent caused reduction. There was no strain which was completely negative to litmus milk. Striking variability in the reaction of \textit{Staph. uberis} in litmus milk has been reported by different workers. Ferguson (1938) mentioned that litmus milk was not reduced before curdling, and that some strains curdled the milk whereas others did not. Seelemann (1939) described the reaction in litmus milk as reddening with neither coagulation nor reduction. Slanetz and Naghski (1940) found acid, acid and reduction, or acid, coagulation and reduction, produced by different strains in litmus milk. Hansen (1935) found only acid production with no curdling in the three cultures examined by him. Plastridge \textit{et al}. (1942) listed acid alone, or acid, coagulation and partial reduction reactions. Little \textit{et al}. (1946) mentioned acid production, curdling, or partial or no reduction in litmus milk. The reaction in litmus milk appeared to be influenced by the dose of inoculum used. It was observed in this study that with three drops of a broth culture from a Pasteur pipette, more strains produced clotting and reduction than when the litmus milk was inoculated by means of a platinum loop. Similar observation was
also reported by Minett (1934) who mentioned that the reactions in litmus milk were variable depending upon the size of the inoculum used. Seeley (1951) supported Minett's observation and added that this fact along with the manner of testing and the history of the culture largely explained the variations in the reports on this matter. Cultures which produced only a reddening of litmus milk when inoculated by a needle, in addition, reduced and curdled litmus milk when the inoculation was made with one drop of the culture. Cullen (1967) found all gradations of reaction in litmus milk, from acid with clotting and reduction, to acid alone. Strains completely negative in litmus milk were not classified as \textit{Str. uberis} as such strains usually had other differences as well.

The results obtained in this investigation showed that 159 (94.6 per cent) out of 168 strains of \textit{Str. uberis} did not grow in milk containing 0.1 per cent methylene blue. Only nine (5.4 per cent) strains showed growth in it. One hundred and fifty-three (91.1 per cent) of them grew in milk containing 0.01 per cent methylene blue. Little (1940) reported that \textit{Str. uberis} coagulated and reduced 1:5000 methylene blue milk and that many strains reduced 1:1000 methylene blue milk. Heeschen and Meyer (1965) reported that 13 (28.2 per cent) of the 46 strains, tested by them, grew in 1:1000 methylene blue milk. Seeley (1951) mentioned that one strain out of 52 grew in 1:1,000 methylene blue milk, the remaining 51 cultures proved resistant. Other workers reported that no growth occurred in milk containing 0.1 per cent methylene blue (Stableforth, 1959; and Cullen, 1967). Slot (1958) mentioned that none of the 156 strains, that he tested, grew in 0.01 per cent methylene blue milk.
As shown in Table 1, different concentrations of methylene blue were used by different workers and consequently variable results of *Str. uberis* reaction were reported. Variation reported by workers using identical concentrations of methylene blue in milk might be due to difference in the bacteriostatic properties of methylene blue from different sources, or it may be due to variation in the dose of inoculum, or previous manner of handling of the culture.

Observations recorded in this study showed that 28 (16.6 per cent) strains out of 168 grew in the presence of 6.5 per cent sodium chloride. Similar results were reported by Slanetz and Naghski (1940) who found that 50 out of 70 strains of *Str. uberis* proved positive in 6.5 per cent sodium chloride broth. Heeschen and Meyer (1965) noticed one out of 46 strains of the organism to grow in this high concentration of salt. Brown and Shuman (1969)\(^1\) observed growth of *Str. uberis* in 6.5 per cent sodium chloride broth, based on a study of 11 strains of the organism. Seeley (1951), however, reported that none of the 52 strains of *Str. uberis*, examined by him, grew in the presence of 6.5 per cent sodium chloride. Similar results were also recorded by Slot (1958). These observations indicate that both sodium chloride sensitive as well as resistant strains of *Str. uberis* exist in nature, though a majority of them are sodium chloride sensitive.

Variable results have been reported by different workers in regard to

the resistance of *Str. uberis* to heat. It was observed in this investiga-
tion that of the 168 strains, 138 grew at 10°C., 161 were able to grow
at 45°C., and 127 survived exposure to 60°C. for 30 minutes. These re-
results are in conformity with those mentioned by Bergey (1957). Slanetz
and Naghshi (1940) recorded similar results of growth at 45°C., but re-
ported that only 10 out of 70 strains, tested by them, grew at 10°C., and
all of them were killed at 60°C. Little (1940) mentioned that *Str. uberis*
was destroyed when broth cultures were heated at 60°C. for 30 minutes.
Slot (1958) and Stableforth (1959) mentioned that *Str. uberis* failed to
grow at 45°C. Slot (1958), however, reported that 141 out of 156 strains
of the organism grew at 10°C. Bergey (1957) stated that some variations
occurred among the individual strains at the two extremes of temperature,
10°C. and 45°C., depending, however, upon the previous manner of handling of
the culture.

Wilson and Miles (1964) mentioned that the heat resistance tests varied
in their reliability and they added that the ability of the organisms to
survive exposure to a temperature of 60°C. for 30 minutes depended upon
such factors as the medium in which they were grown and the number of cocci
present at the start. That seemed to indicate that different readings might
be obtained with the same organism on different days.

The results recorded on the growth of *Str. uberis* on blood agar con-
taining different concentrations of bile salts were in general agreement
with those reported by Seeley (1951). Of the 168 strains, 142 grew in the
presence of 10 per cent bile salts, 102 showed growth on 20 per cent, and
37 were able to grow on 40 per cent bile salts blood agar. The growth in
the presence of 10 and 40 per cent bile salts, together with other charac­
teristics, is an important factor in associating **Str. uberis** with the
enterococcus division of Sherman (1937).

A reaction, reported as specific, for the identification of **Str. agalactiae**
was described by Christie et al. (1944) and was named after
them as CAMP reaction. Their findings were confirmed by Munch et al.
(1945). It was observed that of the 151 strains of **Str. uberis**, isolated
in this study, 17 (11.25 per cent) proved CAMP-positive. Similar results
of CAMP-positive strains of **Str. uberis** were reported by Wilson and Slavin
(1950); Murphy et al. (1952); Hauge and Ellingsen (1953); Slot (1958);
Heeschen and Meyer (1965); and Postle (1968). However, the incorporation
of 0.1 per cent esculin in blood agar, used for the CAMP test, was reported
to help in distinguishing **Str. uberis** from **Str. agalactiae** (Wilson and
Slavin, 1950) in view of its esculin splitting property.

**Str. uberis** does not fit properly in the grouping scheme of Lancefield
(1933), which depends upon precipitation between an extract of the cocci
and a group-specific serum. Of the 151 strains examined, 31 (20.5 per
cent) reacted with Lancefield group E anti-serum. This is in agreement
with the results reported by Plastridge and Williams (1939); Jacob (1947);
Sweeney (1964); Heeschen and Meyer (1965); and Cullen (1966).

**Bacteriophage**

The results recorded on the sensitivity of 147 strains of **Str. uberis**
to 11 phages, isolated from the strains of the organism, revealed that four
of the phages No. 1, II, IV and XI were more active than the other seven. Phage IV lysed the maximum number of strains (118), followed by phage II (91), phage XI (30) and phage I (29). Of the 128 sensitive strains, 126 were lysed by one or more of the four phages. Ninety out of 91 strains, sensitive to phage II, were also sensitive to phage IV. Of the 118 strains lysed by phage IV, 91 were also lysed by Phage II. An analysis of the lytic ability of the four phages showed that of the 126 strains, sensitive to one or more of these phages, all except four were sensitive to phage IV. This seemed to indicate that there was considerable uniformity in the strains of Str. uberis examined. This is in agreement with the biochemical reactions of the organism studied.

The limited number of cultures of enterococci, tested with the four Str. uberis phages, indicated that there was no cross-reaction with group D streptococci. Jacob (1947) contradicted any antigenic relationship between Str. uberis and group D streptococci. However, one out of three strains of Str. pyogenes, and one out of two strains of Str. canis, showed cross-reaction with phage II, and phages II and XI respectively. None of the other 12 cultures belonging to Lancefield groups B, C, E, and L were lysed by any of the four Str. uberis phages.

All the phages grew well at 37°C. but it was observed in this study that even in the presence of confluent lysis on a plate, there was in many cases a thin layer of phage-resistant bacteria. This was particularly observed in the case of phages II and IV. The thin layer covered the entire area of lysis in some cases. A similar phenomenon was reported by Kjems (1955) in the case of group A and group D streptococcal phages. Maxted
also observed thin layer of phage-resistant bacteria in his studies. He found that the appearance of the mucoid colonies, giving rise to the layer, could be inhibited by adding hyaluronidase to the medium in the plates. Kjems (1955) reported that the mucoid colonies failed to appear when the plates were incubated at 22°C., instead of 37°C. This worked well only in the case of his phage I but not with the other three phages where the thin layer invariably remained even in the presence of confluent phage growth. He concluded that phage I was more active at 22°C. than at 37°C.

The above preliminary observations seem to indicate that bacteriophage activity of Str. uberis may assist in differentiating the organism from other streptococci. Though no reference appears to be available on the bacteriophage activity of Str. uberis but such findings have been reported in the case of other streptococci.

It may be mentioned that perhaps too much rigidity was exercised in the isolation of strains of Str. uberis and only those strains which conformed to the accepted normal patterns were generally included in the collection of cultures. It is felt that a more liberal approach might have made a larger number of isolates available for study, thus affording greater opportunity to study the different variations within the species and providing more varied data to correlate different biochemical patterns with phage sensitivity.

Incidence and Ecology

The observations recorded indicated that the udder skin accounted for the greatest percentage of Str. uberis isolates, followed by the teat skin, the two sites contributed 49.64 per cent of all the isolations. On 16 out
of 18 occasions when *Str. uberis* was excreted in the milk, the infection
did exist on the udder and/or teat skin simultaneously. One cow, however,
which had shown infection of the udder skin and milk for two weeks, later
showed only infection of the milk without infection of the udder or teat
surfaces. It appeared that isolation of *Str. uberis* in this case might have
been missed through some error in the isolation procedure or the animal
might have been given some antiseptic wash. The existence of *Str. uberis*
in the milk and on the udder and/or teat skin on 16 out of 18 occasions
did seem to indicate that the infection of the mammary gland was secondary
to infection of the udder and/or teat skin. In some cases, infection of the
udder and teat skin was observed without infection of the mammary gland
parenchyma. These results are in conformity with those of Sweeney (1964)
who mentioned that udder skin was the most important reservoir of *Str.
uberis* and that infection of the mammary gland was invariably secondary to
infection of the udder surface. In one cow excreting *Str. uberis* in the
milk, the organisms were not isolated from the udder or teat surfaces but
were obtained from the lips of the cow. Cullen (1966) reported that lips
were the best site for the survival of *Str. uberis* and that the skin of
the teats was always a comparatively unfavorable site for it. The findings
obtained in this study, except for one cow referred to above, did not agree
with those of Cullen (1966). He added that *Str. uberis* was able to survive
and multiply on the lips, without continuous replenishment from other sources.
For a period of four weeks in August and September it was not recovered
from any other site. The existence of *Str. uberis* infection on the lips
during the period, mentioned by him, did not result in mammary gland in-
fection as the organisms were not recovered from any of the milk samples examined during that period. This seems to suggest that though the organisms survive on the lips, they do not contribute directly to infection of the mammary gland parenchyma but may serve to spread the infection through licking from the udder and teat skin to other parts of the body.

The incidence of *Staph. uberis* infection was found to be the highest in winter (December to February) and the least in summer (June to August). Similar results were reported by Hughes (1960), and Edwards and Smith (1966). Variable results have been reported by different workers in regard to the incidence of infection in different months of the year but most of the workers agree that the infection shows seasonal trends. It is likely that variations in climatic conditions and animal husbandry practices in different countries or in different regions of the same country may be responsible for difference in the month-wise incidence of infection in different areas.

The incidence of *Staph. uberis* infection was found to be highest during the early lactation period as compared to the mid and late lactation, or the dry period. Similar findings were reported by Priestley and Artioli (1945); Watts (1951); and Hughes (1960). Cullen (1966) mentioned that both weather and lactation stage might be important determinants of *Staph. uberis* populations.

Observations recorded on 11 cows showing mammary gland infection indicated that the incidence of *Staph. uberis* infection increased as the cows advanced in age. Five out of six (83.3 per cent) cows in the age-groups from 6-12 years were found infected as against six out of 18 (33.3 per cent)
cows in the 3-6 years age-groups. This is in conformity with the findings reported by Seelemann (1932); Davis and McClemont (1939); Priestley and Artioli (1945); Murphy (1947); Lancaster and Stuart (1949, 1951); Stuart and Lancaster (1949); Livoni (1955); and Edwards and Smith (1966). Ormsbee and Schalm (1949) reported that excluding cows with incompetent sphincter mechanisms, susceptibility to streptococcal mastitis was randomly distributed, regardless and independent of age and previous history of infection. However, degree and duration of exposure to infection and the hygienic measures taken to prevent it have been reported to be factors of prime importance (Stableforth, 1959; McEwen and Samuel, 1946; and Spencer and Kraft, 1949).
SUMMARY

An investigation into the incidence and ecology of *Str. uberis* in 24 dairy cows was carried out for one year. The strains of *Str. uberis*, isolated, were subjected to biochemical and physiological tests in order to characterize the organism. Bacteriophage activity was studied and the strains of *Str. uberis*, isolated, were tested for sensitivity to the phages with a view to explore the possibility of using it for the identification of *Str. uberis*.

One hundred and seventy-two strains of *Str. uberis* were isolated. Of these, 151 strains, together with 17 strains obtained from the University of Pennsylvania were tested using 32 biochemical and physiological tests. All the 168 strains, examined, fermented salicin, mannitol, inulin, trehalose, glucose, maltose, fructose, sucrose, and sorbitol. Lactose was fermented by all but two strains. All of them hydrolyzed sodium hippurate, esculin, and arginine. None hydrolyzed starch.

Of the 168 strains, 82.1 per cent grew at 10°C., 95.8 per cent grew at 45°C., and 75.6 per cent of the strains were able to survive a temperature of 60°C. for 30 minutes. In 4.0 per cent sodium chloride broth, 89.9 per cent of the strains showed growth, and 16.6 per cent were able to grow in the presence of 6.5 per cent sodium chloride. The reaction in litmus milk was quite well marked, a majority of the cultures produced acid, coagulation, and reduction. There was no strain which was completely negative to litmus milk. As many as 91.1 per cent of the strains grew in 1:10,000 methylene blue milk, and 5.4 per cent were able to grow even
in 1:1,000 methylene blue milk. Tolerance tests in the presence of bile salts revealed that 84.5 per cent of the strains grew in 10 per cent, 60.7 per cent in 20 per cent, and 22.0 per cent of them were able to grow in the presence of 40 per cent bile salts. All the strains proved negative for tyrosine decarboxylase activity. Most of the strains (90.5 per cent) were non-hemolytic, 7.1 per cent were alpha-hemolytic, and 2.4 per cent showed narrow-beta type hemolysis.

Of the 151 strains, isolated, 17 (11.25 per cent) proved positive to the CAMP test. Thirty one (20.5 per cent) of them reacted with Lancefield group E antiserum.

There were 19 streptococcal strains which resembled Str. uberis in some characters but were inulin-negative. Each one of them was found to show some other reactions which were not typical of Str. uberis species. They were group E-negative. They were not included in this species.

The sensitivity of 147 strains of the organism to 11 Str. uberis phages was tested. Results obtained indicated that four phages, Nos. I, II, IV and XI, were more active than the remaining seven. Phage IV lysed 118 strains, phage II caused lysis of 91 strains, phage XI lysed 30, and phage I produced lysis of 29 strains. Of the 128 sensitive strains, 126 were lysed by one or more of the four phages. An analysis of the lytic ability of the four phages revealed that of the 126 sensitive strains, all except four were sensitive to phage IV. This indicated that there was considerable uniformity in the strains of Str. uberis studied. This is in conformity with the biochemical reactions of the strains of the organism. It may be mentioned that five inulin-negative strains, tested, were also sensitive
to one or more of these phages. None of the 11 cultures of enterococci, tested with the four *Str. uberis* phages, showed any lysis. Of the 17 cultures belonging to Lancefield groups A, B, C, E, and L, one strain of *Str. pyogenes* and one strain of *Str. canis* showed cross-reaction with phage II, and phages II and XI respectively.

The ecological studies indicated that udder and teat surfaces seemed to be the most important reservoirs of *Str. uberis* as 49.64 per cent of the strains were isolated from them. The infection of mammary gland parenchyma appeared to be secondary to infection of udder and/or teat skin.

The incidence of *Str. uberis* infection was highest in winter, followed by fall, spring, and the least in summer. December recorded the maximum number of isolations and June the least. Observations recorded on 11 cows showing mammary gland infection indicated that the incidence of *Str. uberis* infection increased as the cows advanced in age. Five out of six cows in the age-groups from 6-12 years were found infected as against six cows out of 18 infected in the 3-6 years age-groups. It was observed that the incidence of *Str. uberis* infection was highest during the early lactation period, followed by mid and late lactation period, and the least during the dry period.
BIBLIOGRAPHY


Little, R. B. 1940. Streptococci other than Streptococcus agalactiae found in the cow's udder. Cornell Veterinarian 30: 482-494.


Murphy, J. M. 1946. The genesis of bovine udder infection and mastitis. 3. Discussion of the age factor in streptococcal (Str. agalactiae) infection. U.S. Livestock Sanitary Association Proceedings 50: 119-127.

Murphy, J. M. 1947. The genesis of bovine udder infection and mastitis. II. The occurrence of streptococcal infection in a cow population during a seven-year period and its relationship to age. American Journal of Veterinary Research 8: 29-42.


Packer, R. A. 1943. The use of sodium azide (NaNg) and crystal violet in a selective medium for streptococci and \textit{Erysipelothrix rhusiopathiae}. Journal of Bacteriology 46: 343-349.


Stableforth, A. W. 1937. Serological types of Streptococcus agalactiae (Streptococcus group B) in this and other countries. Journal of Pathology and Bacteriology 45: 263-277.


Stuart, P. and Lancaster, J. E. 1949. Some factors which may be concerned in the susceptibility of the bovine udder to Streptococcus agalactiae infection. Journal of Comparative Pathology and Therapeutics 59: 31-41.


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