A STUDY OF MASTITIS IN DAIRY HERDS WITH PARTICULAR REFERENCE TO *STREPTOCOCCUS UBERIS*

by

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SUMMARY

Streptococcus uberis (*S. uberis*) is the infectious agent in a significant proportion of cases of subclinical mastitis in dairy cows in the British Isles, and in some cases of clinical mastitis. It is a pathogen which can exist outwith the bovine mammary gland parenchyma, having been isolated from bovine skin, ruminal fluid, faeces and bedding. Using DNA typing of individual strains of *S. uberis*, it was hoped that an epidemiological survey of a herd infected with *S. uberis* could resolve whether all or only some strains of *S. uberis* (and *Streptococcus parauberis*) found on cows' skin and in the environment could gain access to the glandular tissue and cause mastitis.

In order to find a farm where *S. uberis* was endemic in the dairy herd, milk and other samples were examined from seven herds in south-west Scotland that were likely to have a subclinical *S. uberis* problem. However, bacterial analysis of the milk samples collected showed that *Staphylococcus aureus* (*Staph. aureus*) was the predominant pathogen causing mastitis in the herds. The infrequent isolation of *S. uberis* from only one of the herds suggested the possibility that the cows' immunological control of *S. uberis* infection was incidental to having to deal with the constant *Staph. aureus* infection and, were the latter resolved, infection by *S. uberis* may become a more noticeable and serious problem.

A protocol was developed to screen a large number of milk samples for the presence of *S. uberis* employing as few biochemical tests as possible, consistent with accurate identification; the tests were fermentation of inulin followed by haemolysis of blood agar and hydrolysis of aesculin and hippurate. In addition, a new "enrichment" broth consisting of equal volumes of Todd Hewitt and tryptose broths was found to produce a greater harvest of *S. uberis* colony forming units than conventional enrichment broths: The use of litmus milk as an enrichment broth was also discussed.

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LIST OF ABBREVIATIONS

BP	British Pharmacopoeia
cfu	colony forming unit
°C	degree centigrade
DNA	deoxyribonucleic acid
g	gram
Gm	gram
mg	milligram
μg	microgram
iu	international unit
l	litre
ml/cc	millilitre/cubic centimetre
μl	microlitre
log	logarithm
cm	centimetre
mm	millimetre
M	molar
mM	millimolar
N	normal
%	per cent
pH	$-\log_{10}$(hydrogen ion concentration)
ppm	parts per million
RNA	ribonucleic acid
w/v	weight to volume
v/v	volume to volume

For clarity *Staphylococcus aureus* has been abbreviated to *Staph. aureus*

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DECLARATION

I, Timothy Richard Barker, hereby declare that the work presented in this Thesis is original, was carried out by me, and has not been presented for an award of a degree in any other University.

I made all the farm visits, analysed all the data and, with the exception of several isolates of *Streptococcus uberis* sent to me by the Scottish Agricultural Colleges' Veterinary Investigation Centres at Inverness and Auchincruive, collected all the samples. With the exception of measurement of milk somatic cell counts by either the Scottish Milk Records Association Cell Count Advisory Service or the Haematology Unit of the Department of Veterinary Pathology, Glasgow University Veterinary School, the laboratory work was carried by me in the Department of Veterinary Medicine of Glasgow University Veterinary School.

CHAPTER ONE

A REVIEW OF THE LITERATURE

1.1 THE EARLY HISTORY OF MASTITIS INVESTIGATION

1.1.1 THE GENESIS OF MILK MICROBIOLOGY

According to Lister (1878), one of the earliest studies of the microbiology of bovine milk was carried out by Pasteur (1858) who noted that "if you examine any specimens of souring milk with the microscope, you will find little organisms".

1.1.2 ROUTE OF ENTRY OF MASTITIS PATHOGENS

Smith and Brown (1915) recorded that bovine mastitis was originally thought to be the result of "exposure, injury, retention of milk and certain disturbances of health". However, the same authors noted that bacteria had been implicated in the disease by Franck (1876) who showed that severe mastitis developed in healthy udders after "injecting putrescent fluids into the duct and by transferring milk from an infected to a normal udder", and by Kitt (1913) who, in 1886, found that certain strains of colonic bacilli caused mastitis after "smearing the bacteria on the opening of the (teat) duct": Smith and Brown also noted that Bang (no date or reference given) obtained the same result "by introducing bacteria (into the teat) on the end of a glass rod". Savage (1909) reported that Nocard and Mollereau (1887) found that healthy bovine quarters developed mastitis after being infused with pure cultures of streptococci that had been isolated from milk collected from quarters affected with mastitis.

Such findings led to the general acceptance that the teat canal was the route of access for infectious agents and that "some of the germs found in the normal udder might well be derived from the bedding-litter, the fodder, the faeces and perhaps most importantly of all, the hand and person of the milker" (Henderson, 1904). However, Jones (1918a) reported that Steiger (1904) had thought that infection by "metastasis" through the blood stream or lymph system was possible, and also that Guillebeau and Hess (no date or reference given) had found that when *Bacillus coli* (*B. coli*) was injected subcutaneously into goats, it became localised in the mammary gland. Later, the haematogenous route of udder infection was dismissed by Savage (1909) on the grounds that mastitis causing organisms that were "inoculated" into one half of a goat's udder could never be isolated from the contralateral uninoculated half and that, by inference, if the blood stream or lymph system were routes of infection, any successful invasion by pathogenic bacteria would cause simultaneous infection of both halves of a goat's udder and all four quarters of a cow's udder.

1.1.3 TYPES OF MASTITIS

Smith and Brown (1915) reported that Bang (1889) had found that different species of bacteria produced different types of inflammation in the udder: Streptococci isolated from a case of bovine mastitis and introduced into the milk duct of another cow produced "a slowly progressive, chronic catarrh leading to complete atrophy of the glandular tissue", streptococci from a case of equine strangles caused a "severe, acute, purulent inflammation ending in atrophy", and staphylococci gave rise to "acute inflammations, both rapid and slow, leading eventually to recovery".

Kitt (1913) classified the aetiological agents of mastitis into the coli-bacterial group, the paratyphoid and enteritis groups, the staphylococci group and the streptococci group and, according to Jones (1918a), he described the many forms of the gross pathology of

the disease as catarrhal, parenchymatous and purulent inflammation, abscess formation, sclerosis, and a general rapid necrosis of the mammary tissue.

1.1.4 EARLY INVESTIGATIONS INTO THE BACTERIOLOGICAL AETIOLOGY OF MASTITIS

One of the earliest investigations into the bacteriological aetiology of bovine mastitis was carried out by Nocard and Mollereau (1884) who reported "Examined a great number of preparations following Erlich's (*sic*) method, this milk did not present any trace of the bacillus of Koch; the infection of the udders was not, therefore, a case of tuberculosis. On the other hand, on all the slides a special organism was visible in considerable numbers, unique in that it was cultivated in milk in its pure state, this organism took the form of a long string (French text "chapelet": string of beads, rosary, string of sausages/onions), very regular in shape, of very small dimensions, each grain of which, slightly ovoid and measuring 1μ in width and 1μ¼ (*sic*) in length, firmly fixed the colours derived from aniline (vesivine, methylene blue, fuchsin, gentian violet, etc)". Three years later, the same authors isolated streptococci from the milk of cows suffering from mammitis (inflammation of the mammary gland), and they described the bacteria as growing as long chains in bouillon (broth) and able to ferment glucose, lactose, cane sugar and mannite; staining the bacteria by Gram's method, however, gave "mediocre results" (Nocard and Mollereau, 1887).

Once the bacterial aetiology of mastitis was recognised, reports of the bacterial species isolated from cases of mastitis began to be published. Ten organisms were detailed in a survey of 86 cases of infections of the mammary gland (Guillebeau, 1890); *Staphylococcus mastitis* (*Staph. mastitis*) was isolated from 33 of the cases, with three species of *Galactococcus* being isolated from a further 19 cases. Jones (1918a) reported that the three sub-species of *Bacillus guillebeau* (Freudenreich) isolated from

another 22 of the cases were later identified as *B. coli* by Jensen (1897). *Streptococcus mastitis sporidicae* (*S. mastitis sporidicae*) was isolated from eight of the remaining 12 cases, and *Streptococcus mastitis contagiosae* (*S. mastitis contagiosae*) (Nocard and Mollereau) from three cases. The remaining infection was caused by a species named *Chlorobacterium lactis*. A similar range of organisms were isolated by Steiger (1904) from 45 cases of mastitis in cows and one case in a goat.

1.1.5 THE BACTERIA OF "NORMAL" MILK AND UDDER TISSUE

In the early years of the study of bacteriology it was thought that milk, freshly drawn from the udder, was sterile and would keep indefinitely so long as outside contamination was prevented (Evans, 1916). Henderson (1904) noted that according to Uhlmann (1903a) many workers including von Freudenreich and Ward (no dates or references given) had subscribed to the view of milk being "germ free" and that Kitt (1903) had stated that "the normal milk inside the udder, and at the moment of milking, is absolutely free of micro-organisms". Evans (1916) credited Schulz (1892) with being the first to show that in freshly drawn milk "the first milk to be drawn contained large numbers of bacteria and that the numbers decreased as the milking progressed".

Savage (1908) reported that Ward (1900) "examined the udders of 19 freshly slaughtered milch cows, and found that the lactiferous ducts of all 19 harboured bacteria throughout their whole extent". In the same paper, Savage (1908) also noted the work of Uhlmann (1903b) who, after sectioning the teats of 35 cows, found bacteria "mostly only a few, occasionally hundreds" in all the sections.

The bacterial content of the glandular tissue of fourteen udders affected with mastitis was compared with that of seven "normal" udders by Henderson (1904), all the udders having been removed from freshly slaughtered cows. The udders were sectioned and

"cultures on various media were made from the tissues". Bacteria were isolated from the sections on "cover glass films direct from the tissues" but there was no record of milk samples being collected for bacteriological examination. Although the author suspected the presence of bacteria in the normal udders, "the actual finding of them came somewhat as a surprise"; this finding was explained by the micro-organisms being "almost exclusively confined to the walls of the ducts and that they exist there as epiphytes rather than as true parasites" (Henderson, 1904).

Evans (1916) reported that, according to de Freudenreich (1904), bacteria that multiplied in the udder and were able to withstand the bactericidal properties of the milk and tissues "appear to be quite inoffensive". Some years later, when discussing whether bacteria found in cases of mastitis "should be regarded as pure parasites or more properly as normal inhabitants of the udder which for some reason or other have temporarily increased in numbers", Minett, Stableforth and Edwards (1929) stated that although streptococci were encountered in the "normal" udder, they could not be taken to be normal inhabitants "since there is no denying the facts that in most quarters in which these streptococci can be demonstrated, pathological disturbance does arise sooner or later".

1.1.6 GENUS STREPTOCOCCUS

According to Jones (1978), the term 'streptococcos' (*sic*) was first used by Billroth (1874) to describe chain forming coccoid bacteria that he isolated from various wounds and discharges, and from about half the cases of erysipelas that he attended. Both Andrewes and Horder (1906) and Brown (1919) noted that within a decade of Billroth's publication, 'Streptococcus' was used as a generic name by Fehleisen (1883), who gave the name *Streptococcus erysipelatos* (*S. erysipelatos*) to bacteria isolated from cases of erysipelas, and by Rosenbach (1884), who gave the name

Streptococcus pyogenes (*S. pyogenes*) to an organism that he isolated from suppurating lesions in man. After considerable debate, the two species *S. erysipelatos* and *S. pyogenes* were considered to be synonymous (Andrewes and Horder, 1906; Brown, 1919).

1.1.7 'STREPTOCOCCUS BREVIS' AND 'STREPTOCOCCUS LONGUS'

Andrewes and Horder (1906) reported on an early classification of the species of streptococci that had been proposed by von Lingelsheim (1891) who "suggested the terms 'streptococcus brevis' for the short-chained forms rendering broth uniformly turbid and for the most part non-virulent, and 'streptococcus longus' for the long-chained forms, those in which the long chains settle down as a flocculent or granular precipitate, leaving the supernatant broth clear....the majority of virulent forms belong to this latter type". In a medical report to the Local Government Office for 1907 to 1908 (Savage, 1909), great emphasis was put on chain length as a means of distinguishing the various species of streptococci even though the same report also cited the findings of Stäheli (1904) who had concluded that the length of the chains and the size of the individual cocci were very inconsistent and, therefore, unsuitable as a basis for classification. This latter opinion was subsequently endorsed by Andrewes and Horder (1906) who stated that varying lengths of chain could be found in the same pure culture, and that chain length varied with the culture medium employed and the degree of cohesion between the constituent cocci. Later, Brown (1919) also dismissed the findings of von Lingelsheim "not only because it is a matter of common laboratory experience that length of chain is readily influenced by conditions of cultivation, but because *S. longus*, defined by von Lingelsheim (1899) as including those streptococci which have a tendency to grow in six or more elements, includes practically all streptococci".

1.1.8 *STREPTOCOCCUS LACTIS (LACTOCOCCUS LACTIS)*

Lister (1873; 1878) isolated and described a bacterium that caused milk to sour and gave it the name *Bacterium lactis*. However, Orla-Jensen (1919) reported that the same organism subsequently appeared in the literature as *Streptococcus acidi lactici* (Grotenfeld, 1879 - no reference given), *Bacterium lactis acidi* (Leichmann, 1894 - no reference given), *Bacterium Güntheri* (Lehmann and Neumann, no date or reference given) and *Streptococcus lacticus* (Kruse, no date or reference given). Orla-Jensen (1919) preferred the name *Streptococcus lactis* (*S. lactis*) as, by then, it was realised that the cells were coccoid or spherical although "somewhat longer than they are broad" and not, as earlier thought, a rod shaped bacterium. This latter classification of the bacterium was used throughout most of the twentieth century, credit being given to Löhnis (1909) for the introduction of the name *S. lactis* (Mundt, 1986a). However recent work, using nucleic acid hybridisation techniques, have shown that *S. lactis*, together with three minor species of *Streptococcus* and two species of *Lactobacillus*, are closely related to each other, but not to other streptococci (Schleifer, Kraus, Dvorak, Kilpper-Bälz, Collins and Fischer, 1985); it has been proposed, therefore, that these species should be transferred to a new genus *Lactococcus*, with *S. lactis* being renamed *Lactococcus lactis* (*L. lactis*) (Schleifer *et al*, 1985; Schleifer and Kilpper-Bälz, 1987).

1.1.9 CLASSIFICATION OF STREPTOCOCCAL SPECIES BY BIOCHEMICAL TESTS

By the end of the nineteenth century, differentiation of streptococcal isolates by their ability to ferment various substrates was already established, although the methods and apparatus were somewhat cumbersome (Durham, 1898). Durham (1898) reported on a modified apparatus consisting of a test tube that had an inverted miniature test tube (Durham tube) placed in it. The whole apparatus with the substrate *in situ* was steamed

for 20 minutes on three consecutive days; this process not only sterilised the substrate, but caused it to fill the inverted Durham tube. If the substrate was then inoculated with a streptococcal isolate and incubated, a bubble of gas trapped at the closed end of the Durham tube indicated that the substrate had been fermented. In addition, Durham found that the resulting increased acidity of the medium could be shown by the reduction of litmus indicator that had been added to the substrate before incubation.

Gordon (1905a) found that "saliva streptococci" isolated from "vitiated air passing out of the Debating Chamber of the House of Commons" could be differentiated from isolates of an "air streptococcus" by means of "minor morphological points of distinction", and more significantly by the fact that "the air streptococcus produced no change of reaction and no clot when growing in the litmus milk, and also failed to change the colour of the medium when growing in neutral red broth". Reactions of the streptococci in two per cent solutions of either glucose or lactose added to slightly alkaline, sugar free broth, were also different; whilst all the examples of salivary streptococci produced an acid reaction in both glucose and lactose broths, the air streptococci only fermented the glucose substrate.

A further twelve streptococcal isolates, collected from various sources, and tested on a wider range of organic substrates, coniferin, dulcite, fructose, galactose, inulin, maltose, mannite, mannose, raffinose, saccharose, salicin and starch, as well as glucose and lactose, produced many different fermentation patterns (Gordon, 1905a,b). After experimenting with a total of 33 substrates, seven of them, namely the two disaccharides saccharose and lactose, the trisaccharide raffinose, the polysaccharide inulin, the two glucosides salicin and coniferin, and the alcohol mannite, were considered useful for routine differentiation, with additional substrates employed as necessary.

Using Gordon's biochemical tests, Andrewes and Horder (1906) isolated and described six streptococcal species: *Streptococcus equinus* "derived chiefly from air, dust and horse dung" and also from human saliva, faeces and urine, *Streptococcus mitis* "essentially saprophytic, occurring chiefly in human saliva and faeces", *Streptococcus salivarius* found in saliva, *Streptococcus faecalis* (*S. faecalis*) isolated from human intestinal contents, *Streptococcus anginosus* (*S. anginosus*) isolated from human sore throats and cases of scarlet fever, and *S. pyogenes*. The authors reported their isolates of *S. pyogenes* as being identical to the *S. pyogenes* described by Rosenbach (1884) and, in addition, they stated that although the name *S. erysipelatos* given by Fehleisen had "undoubted priority, this species does so much more than produce erysipelas that the wider term (*S. pyogenes*) deserves to supplant the narrower". The species *S. anginosus* was later renamed *Streptococcus milleri* (*S. milleri*) by Guthof (1956) (Hardie, 1986; Schleifer and Kilpper-Bälz, 1987).

Fermentation tests were later used in the definition of *Streptococcus faecium* (*S. faecium*) "the most frequently found streptococcus in the human faeces", and *Streptococcus bovis* (*S. bovis*) "the most common streptococcus in cow dung" (Orla-Jensen, 1919).

1.1.10 THE STREPTOCOCCI ISOLATED FROM BOVINE MILK

The medical report to the Local Government Office for 1906 to 1907 (Savage, 1908) detailed a study, which included the use of Gordon's fermentation tests, to find which species of streptococci and other cocci were present in the milk of "reputedly healthy cows" whose udders and teats were "clean to the naked eye". With the exception of the foremilk being drawn off, no presampling antiseptic precautions were taken and the teats were not washed before the milk samples were collected. The pellets obtained from centrifuging the milk samples were inoculated on to nutrient agar and, after incubation at 37°C for 24 or 48 hours, potential streptococcal colonies were subcultured in nutrient

glucose broth or neutral red glucose broth, and also on nutrient agar. If microscopic examination showed the isolates to be streptococci, their fermentative action on coniferin, lactose, mannite, raffinose, saccharose and salicin was determined; the fermentative action on what the author referred to as indol in the text, but as inulin in the tables of results, was also determined. No attempt was made to identify or classify the various streptococci tested.

In the medical report to the Local Government Office for the following year (1907 to 1908), Savage (1909) recorded that "the organism described by Nocard, that by Adametz under the name *S. agalactiae contagiosae*, by Guillebeau as the *Streptococcus mastitidis sporadiae*, and the streptococci of other observers were culturally so little defined that they would include not only all the streptococci found in mastitis cases, but many of the streptococci of healthy milk" (no dates or references given). The author was of the opinion that even with the considerable diversity of morphological, cultural and pathological characteristics found in the streptococci, it was probable that 80% of the streptococci associated with mastitis were of "one very definite type", and he proposed that the name *Streptococcus mastitidis* (*S. mastitidis*) be reserved for organisms of that type and origin. It should be noted that Steiger (1904) had lengthened the term *'mastitis'* in Guillebeau's nomenclature of *Staph. mastitis*, *S. mastitis sporidicae* and *S. mastitis contagiosae* to *'mastitidis'* in both staphylococcal and streptococcal species. The term *'mastitidis'* continued to be used by some authors (Savage, 1909; Jones, 1918a; Ayers and Mudge, 1922), although credit for the amended names was still given to Guillebeau.

Although Kitt (1893) used the name *Streptococcus agalactiae contagiosae* for the streptococcus of bovine mastitis (Haupt, 1952), which Lehmann and Neumann (1896) subsequently shortened to *Streptococcus agalactiae* (*S. agalactiae*) (Hansen, 1953), it was many years before this name became generally recognised (Judical Commission for

Bacteriologial Nomenclature and Taxonomy, 1954), credit for the name being given to Lehmann and Neumann; however, the commission recognised that "the description of the characteristics of the species as given by Lehmann and Neumann are probably inaccurate in some particulars, and differ from the descriptions of earlier writers".

1.1.11 ZOONOTIC IMPLICATIONS OF INFECTED MILK IN HUMAN DISEASE AND PUBLIC HEALTH

The primary stimulus for scientific research into bovine mastitis came from the need to clarify as to whether disease epidemics in the human population could be disseminated by the drinking of infected milk (Smith and Brown, 1915; Jones, 1918b): Although there had been convincing epidemiological evidence, in no instance had there been bacteriological proof (Savage, 1908). *Streptococcus mastitidis*, the species most commonly isolated from cases of bovine mastitis, and *S. anginosus* (*S. milleri*), the organism most frequently isolated from human sore throats, were indistinguishable morphologically and culturally, and both gave identical carbohydrate fermentation reactions (Savage, 1909). However, when injected into small laboratory animals, *S. mastitidis* produced only occasional local abscesses whilst *S. anginosus* (*S. milleri*), and also *S. pyogenes*, showed considerable pathogenic effect which, in mice, was frequently lethal. Smith and Brown (1915) reported that, although constrained not to inoculate other human throats with mastitis-causing streptococci to see if inflammation resulted, "Savage inoculated his own throat with an abundance of streptococci from two cases of mastitis in cows. Neither inoculation was followed by symptoms of any kind and the inoculated streptococci disappeared very rapidly". Streptococci isolated from human throats never caused mastitis when inoculated into goats' streak canals, whilst streptococci isolated from the ulcerated teat of a cow caused marked mastitis in goats (Savage, 1909).

Haemolytic streptococci, isolated from infected human tonsils and smeared both directly, and after incubation in milk, on to the teats of a cow for three days in succession, did not result in mastitis in the corresponding quarters (Davis and Capps, 1914), or raise the number of leucocytes in the milk from those quarters, the latter being counted by the Doane-Buckley method (Doane, 1905). However, applying the same material to an abrasion made "near the meatus" of the teat did result in mastitis in the corresponding quarter, as did infusing the culture "into the udder by means of a catheter inserted to a distance about 8cm above the meatus". From this evidence, Davis and Capps concluded that it was possible for haemolytic streptococci, from the contaminated hands of a milker suffering from a sore throat, to enter a teat and cause mastitis "lasting for several weeks in cows. This time roughly corresponds to the duration of milk-borne (human) epidemics".

Continuing public concern over the unresolved threat of enzootic infection by bovine mastitis led to a study of the organisms found in the milk of cows in a large dairy herd where mastitis was endemic (Jones, 1918a,b,c,d). In addition to the need to determine if there was a connection between bovine mastitis and human tonsillitis epidemics, the study also aimed to reduce the high cost of the infection that was being borne by dairy farmers. The size of the herd was not reported, but in one year (1916) over 60 cows had to be "disposed of" due to chronic mastitis, this being double the number of cows "reacting to tuberculin"; many other animals developed milder forms of mastitis (Jones, 1918a). In 1917, mastitis was responsible for the loss of a further 71 cows (Jones, 1918d). Although detailed records were kept on each cow and various tests performed on the milk samples (bacterial count, haemolysis, fermentation tests, serological agglutination tests, somatic cell counts), no specific identification of the various streptococcal species was attempted.

The same study also sought to find how the "virus" was transmitted from infected to uninfected cows in the hand milked herd (Jones, 1918d). After tests that involved a milker's hands being disinfected in a weak solution of mercuric chloride (concentration 1:1,000) for two minutes between milking each cow, it was concluded that the organism could be passed from cow to cow on the hands and under the finger nails of a careless milker. No work was carried out to see whether the milking utensils and udder cloths could be responsible for the transmission of infection as this was considered unlikely.

Six years later, Ayers and Mudge (1922) concluded that *S. mastiditis* was commonly found in the udders of cows but, in view of the concern for human health, they stated that "the fact that milk containing these organisms has been consumed regularly with no ill effects indicates that these streptococci need not be feared".

1.1.12 MASTITIS AND POST-CALVING UTERINE INFECTION

Prompted by the suggestion of Williams (1918) that mastitis often followed post-calving retention of the placenta and metritis, Jones (1918d) recovered 34 strains of non-haemolytic streptococci from the vaginas of 64 cows, 50 of which were known to have had a history of regular clinical mastitis; the remaining 14 had "recently aborted or passed through normal parturition". Only two streptococcal strains were found to have characteristics in common with streptococci associated with mastitis.

1.1.13 THE PATHOGENICITY OF STAPHYLOCOCCAL SPECIES

The apparent mildness of the cases of mastitis from which staphylococci were isolated gave rise to doubt as to whether this bacterial species was the aetiological agent as "these organisms were present in practically every milk examined.....even when drawn direct from the udder" (Savage, 1908): "In general they are probably without significance, but

they have this importance, that unless their normal presence is recognised, a causal significance may be incorrectly attached to their detection in slightly abnormal conditions".

Most cases of staphylococcal mastitis studied by Jones (1918c) were found to be mild "with only a slight inflammation of the udder. The infected quarter may be slightly enlarged, a trifle firm and secrete flocculent milk for a day or two". However, other cases showed initial acute inflammation that subsided to a chronic condition with atrophied quarters yielding a small amount of purulent secretion for several months. Staphylococci constituted the majority of the micrococci isolated from the udder secretions, being differentiated from the galactococci by their ability to liquefy gelatin. The staphylococcal isolates varied in the amount of acid they produced from the fermentation of dextrose, inulin, lactose, maltose, mannite, raffinose, saccharose, and salicin. It was thought that "micrococci similar in many respects to those associated with mastitis have been found to occur in the normal udder", and that they belonged to the normal flora (Jones, 1918c).

1.1.14 HAEMOLYSIS OF BLOOD AGAR BY STREPTOCOCCI

According to available evidence, the ability of some species of streptococci, growing in a blood agar medium, to produce one or more concentric rings of partially or fully haemolysed blood around each bacterial colony was first described by Schottmüller (1903). Some years later, in a study to distinguish the agent causing outbreaks of human tonsillitis from the common throat coccus and to ascertain the role of milk as a carrier of human infections (Smith and Brown, 1915), the patterns of the haemolytic rings were recognised as being an important aid in the differentiation of various species of streptococci. It was already known that, compared with the usual sporadic tonsillitis in humans, the tonsillitis associated with drinking milk was of a more virulent type,

with the infection disseminating to other organs and tissues. The various streptococcal colonies isolated from the epidemics of human tonsillitis in the State of Massachusetts, United States of America (USA) from 1911 to 1914 were considered to be "all alike in that the colonies produce, immediately around them, a clear hemolyzed area on blood agar plates. They differ from the common throat coccus, our alpha type, in which the colony has a partly discoloured and hemolyzed mantle between it and an outer narrow clearer zone" (Smith and Brown, 1915). This study of the haemolytic properties of streptococcal isolates from bovine mastitis produced no satisfactory evidence that these organisms were the cause of tonsillitis in man, but there remained a possibility that certain strains of virulent human streptococci, inoculated into the udder of a cow, would be able to multiply in the milk ducts and be shed into the milk.

The haemolytic properties of streptococci grown in blood agar were later studied in detail (Brown, 1919), and this work formed the basis for the systematic differentiation of streptococcal species by the type of haemolysis they produced when subcultured on blood agar. The partial haemolysis of the blood corpuscles producing a pale opaque ring around the colony was termed alpha haemolysis, whilst the complete haemolysis of the blood corpuscles that left a clear transparent ring was termed beta haemolysis; the absence of any haemolysis was ambiguously described as gamma haemolysis. Brown (1919) also noted that some streptococcal species gave a greenish colour to the agar surrounding the colonies and included these species in the alpha haemolytic group. However, Ayers and Mudge (1922) subsequently found that many such strains did not produce sufficient alpha haemolysis for them to be included in that group. The "green producing" species of streptococci were later described as "viridan type" (Minett *et al*, 1929).

The zones of haemolysis surrounding colonies of pathogenic beta haemolytic streptococci of human origin were of uniform size and character, whilst those of bovine

strains showed haemolysis of considerable variety (Brown, 1920). Other workers reported that the zone of haemolysis surrounding colonies of streptococci of human origin was larger than that around the colonies of streptococci isolated from fresh bovine milk, and that the haemolytic activity of the human streptococci was approximately 100 times greater than that of the milk streptococci (Ayers and Rupp, 1922); no explanation was given as to how the activity was measured.

1.1.15 THE EFFECT OF TEMPERATURE ON BACTERIAL GROWTH

Davis and Capps (1914) found that the growth of streptococcal cultures, isolated from an infected udder and incubated in milk, differed with temperature and according to whether the culture was pure or contaminated by the "ordinary contaminating bacteria found in milk when no special precautions are taken in the process of milking. They include the *Streptococcus lacticus*, colon bacilli, etc, organisms which ordinarily are not pathogenic". At room temperature (20° to 22°C), the bacterial count of the pure streptococcal culture increased gradually over the first six hours, and had multiplied sixfold after 22 hours. Incubation at 37°C gave an eightfold increase in three hours. When the same cultures were incubated at 20° to 22°C in milk in the presence of contaminating bacteria, the contaminants rapidly outgrew the streptococci after 22 hours and the situation remained unchanged at 48 hours. However, during the initial 22 hours at 37°C, the contaminating bacteria multiplied at a much lower rate compared with the streptococci, but after 48 hours the situation had reversed with the streptococci dying off and the contaminating bacteria growing rapidly.

1.1.16 THE LIMITING HYDROGEN ION CONCENTRATION OF STREPTOCOCCAL ISOLATES

The limiting hydrogen ion concentration reached by isolates grown in broths containing one of a range of fermentable sugars, cane sugar, glucose, inulin, lactose, mannite and raffinose, were measured and found to be a means by which streptococci from various bovine and human sources could be differentiated (Ayers, 1916). Twenty-six of the 34 streptococcal cultures isolated from various unspecified human infections reached a pH of no lower than 5.5, whereas all 18 unspecified streptococcal isolates from human mouths (presumably considered to be normal flora), 79 of 89 isolates from bovine milk and 54 of 60 isolates from bovine faeces, mouths and udders (no further details given) reached a pH of below 4.8. Twenty-six isolates with the weaker limiting hydrogen ion concentration, and hence higher final pH (5.4 to 6.0), recovered from human infections were all beta haemolytic whilst the majority of all other isolates collected from human mouths and bovine faeces, mouths, udders (no further details given) and milk were not (Ayers, Johnson and Davis, 1918). It was considered that "pathogenic streptococci" (presumably pathogenic to man) would act likewise and hence have a higher limiting pH than non-pathogenic streptococci.

When grown in a one per cent dextrose broth, a distinct and constant difference existed in the final hydrogen ion concentration of "*Streptococcus haemolyticus*" (presumably beta haemolytic streptococci) recovered from human and bovine sources (Avery and Cullen, 1919). Of 124 strains from known human origin, 116 reached a final hydrogen ion concentration of pH 5.0 to 5.3, and the remaining eight a pH of between 4.8 and 5.0. Of another 45 isolates, of which 26 were recovered from bovine milk and udders infected with mastitis and 19 obtained from cream cheese, 40 isolates reached a pH of between 4.3 and 4.5, and the remaining five, of which two were of known human types and three of uncertain origin, had a final pH of between 5.0 and 5.2.

1.1.17 DIFFERENTIATION OF STREPTOCOCCAL SPECIES BY THE SODIUM HIPPURATE TEST

Available evidence records that hydrolysis of sodium hippurate by certain bacteria was first seen by van Tieghem in 1864 (Ayers and Rupp, 1922; Hajna and Damon, 1934). Following this observation, various bacilli and cocci were shown to be able to hydrolyse hippuric acid (as the sodium salt) into benzoic acid and glycocoll (Ayers and Rupp, 1922).

$$\underset{\text{hippuric acid}}{C_6H_5CONHCH_2COOH} + \underset{\text{water}}{H_2O} \longrightarrow \underset{\text{benzoic acid}}{C_6H_5COOH} + \underset{\text{glycocoll}}{NH_2CH_2COOH}$$

When "haemolytic" (presumably beta haemolytic) streptococci were grown in a peptone-pepsin medium that contained one per cent sodium hippurate, the streptococci of human origin did not split the hippurate whilst, with the exception of some alpha haemolytic isolates, those from bovine sources did (Ayers and Rupp, 1922); the hydrolysis was completed within two days. Cultures that did not grow well in the peptone-pepsin medium showed better growth in a beef infusion broth. The sodium hippurate broth of Ayers and Rupp was later found to give contradictory and misleading results due to the variability of the particular batch of peptone used in its composition (Coffey and Foley, 1937); when the peptone was replaced with asparagine, consistent results ensued.

Ayers and Rupp (1922) also detailed a test using ferric chloride to show whether the hippurate had been hydrolysed; this was based on the fact that "the protein, the hippurate, and the benzoate are precipitated by ferric chloride, the difference between them being that the protein and the hippurate precipitates are more readily soluble in an excess of ferric chloride than the benzoate". The test protocol was detailed as "0.5cc of a 7% solution of ferric chloride is added to 2cc of the medium. When it is thoroughly

shaken, an insoluble precipitate remains in the mixture if the hippurate has been split into benzoate and glycocoll, whereas the mixture becomes clear on standing five or ten minutes if the hippurate has not been hydrolysed", any precipitate of protein and/or hippurate quickly settling to the bottom of the container. In order to reduce the possibility of a precipitate of phosphate of iron giving a false positive result, "a small amount" of hydrochloric acid was added to the ferric chloride solution to redissolve any iron phosphate formed. The addition of excess ferric chloride had no effect on the benzoate precipitate but caused the hippurate precipitate to redissolve.

In addition to recommending the sodium hippurate medium of Coffey and Foley (1937), Cullen (1967) standardised the formulation of the ferric chloride solution of Ayers and Rupp (1922) by stating that the smallest amount of ferric chloride solution (0.7g of ferric chloride dissolved in 100ml of two per cent hydrochloric acid) that redissolved the precipitate of a control sample of sterile uninoculated sodium hippurate medium was the correct quantity to be added to the test samples.

Thirst (1957) incorporated agar to form a solid hippurate culture medium that was employed to help differentiate *Klebsiella* species from *Cloaca cloacae* (*C. cloacae*).

1.1.18 DIFFERENTIATION OF STREPTOCOCCAL SPECIES BY THE AESCULIN HYDROLYSIS TEST

Available evidence suggests that the ability of some species of bacteria to hydrolyse aesculin was first utilised to identify *B. coli* in samples of water and milk (Harrison and van der Leck, 1909a,b), the presence of the bacterium implying faecal contamination of the liquids. Hydrolytic fermentation of aesculin, a glucoside, produces glucose and aesculetin.

$$C_{15}H_{16}O_9 + H_2O \longrightarrow C_6H_{12}O_6 + C_9H_6O_4$$

Aesculin Water Sugar Aesculetin

In the original tests the aesculetin then combined with iron citrate, already present in the medium, to form a "dark brown salt" (Harrison and van der Leck, 1909a). However, in addition to aesculin, the substrate also contained sodium taurocholate and hence only bile salt tolerant bacteria such as *B. coli* would be able to hydrolyse the aesculin.

Later studies showed that *Micrococcus* (*Staphylococcus*) *aureus* and some *Bacillus* species would not grow after being stained with gentian violet whilst, after contact with the dye for one hour, other species of *Bacillus* "grew as promptly and as luxuriantly as organisms which had not been exposed to the dye" (Churchman, 1912). The behaviour of bacteria to anilin dyes corresponded in general to their reaction to the Gram stain (Krumwiede and Pratt, 1914), however some streptococcal strains were more resistant to the bactericidal action of aniline dyes than other Gram-positive organisms.

According to Edwards (1933), Haxthausen (1927) isolated streptococci from skin samples by incubating them in glucose broth to which crystal violet had been added. A medium that allowed maximum growth of streptococci from milk samples but inhibited the growth of staphylococci (Edwards, 1930) consisted of a 1:20,000 concentration of crystal violet in meat infusion broth containing one per cent glucose and five per cent serum. The size of the streptococcal colonies growing on a blood agar medium containing crystal violet became smaller as the concentration of crystal violet in the medium was increased (Edwards, 1933), although the actual number of colonies did not diminish; increasing the concentration of the dye also caused narrowing of the width of the haemolysed zone surrounding colonies of beta haemolytic streptococci. Unfortunately, organisms such as *S. lactis*, *B. coli* "and a variety of staphylococcus" were not inhibited. Potassium tellurite, which was added to inhibit growth of *B. coli*,

haemolysed the blood agar, and inclusion of mannite, which only the dye resistant streptococci fermented, was of little use as no indicator produced sufficient colour change to the colonies to allow satisfactory differentiation. Eventually it was found that when 0.5g of both aesculin and ferric citrate, and two cubic centimetres (cc) of crystal violet were added to one litre of meat extract agar and 50cc defibrinated blood, the non-streptococcal colonies, growing on the medium, that were resistant to the presence of crystal violet could be distinguished by the black colouration of the colonies. However, the blood agar medium tended to become discoloured and the growth of the streptococci were adversely affected. Better results ensued by leaving out the ferric citrate and doubling the amount of aesculin; the black colour of the non-streptococcal colonies was not so intense but there was no discolouration of the surrounding medium. This medium was later modified by the addition of thallous sulphate, which was found to inhibit organisms other than streptococci (McKenzie, 1941), and the resulting 'Modified Edwards Medium' became, and has remained, the definitive selective medium for the isolation of aesculin hydrolysing streptococci.

1.1.19 DIFFERENTIATION OF STREPTOCOCCAL SPECIES BY THE INULIN FERMENTATION TEST

Streptococcal fermentation tests, using various organic substrates, were normally carried out in a liquid medium, for example five per cent serum peptone water (Cullen, 1967). More recently, substrates have been incorporated in an agar medium so allowing individual bacterial colonies to be isolated and subcultured.

Inulin fermenting streptococci, subcultured on an agar medium containing inulin, crystal violet, thallium acetate, bromocresol purple and bovine serum (Pugh and Bramley, 1977) and incubated at 37°C for up to 48 hours, appeared as grey or yellow colonies of less than 1.5mm diameter surrounded by a yellow zone of reduced bromocresol purple

(Bramley, King and Higgs, 1979). This medium was later modified by substituting the bovine serum with meat extract, yeast extract and bacteriological peptone (Bramley, 1982); inulin fermenting organisms grew on this medium as yellow colonies.

1.1.20 DIFFERENTIATION OF STREPTOCOCCAL SPECIES BY SEROLOGICAL TESTS

The serological characteristics of streptococcal isolates were reported by many workers, including Smith and Brown (1914) and Jones (1918a,b), in the first part of the twentieth century. When precipitation and slide-agglutination tests were used to analyse the serological characteristics of mastitis producing streptococci (Stableforth, 1932), strains of the same serological type were found to possess similar biochemical properties whatever type of haemolysis they produced, whilst strains with similar biochemical properties were not necessarily of the same serological type.

Lancefield (1933) differentiated and grouped one hundred and six strains of "*Streptococcus haemolyticus*" using a serological precipitin test; the strains were isolated from a wide variety of sources including man, other animals, and dairy products, but they were not further identified by biochemical or other tests. Lancefield's system of serological grouping has remained a valuable tool for the identification of strains of streptococci. Ten strains of a group that were referred to as "*Streptococcus viridans*", presumably so called because colonies growing on blood agar were surrounded by a greenish, viridans zone, were not able to be typed by this system. Unfortunately, the problem of serological untypability of species of viridans streptococci, such as *Streptococcus uberis* (*S. uberis*), remains to this day despite attempts by several workers including Plastridge and Williams (1939) and Cullen (1967) to resolve it.

1.1.21 CULTURE AND ENRICHMENT MEDIA FOR STREPTOCOCCI

1.1.21a Todd Hewitt Broth

The development of Todd Hewitt Broth arose from a need to obtain antigenic streptococcal haemolysin for use in titrating the levels of antihaemolysin in the sera of normal or immunised animals and of humans suffering various pathological conditions caused by (beta) haemolytic streptococci, for example rheumatic fever and scarlet fever (Todd, 1932a,b).

When (beta) haemolytic streptococci were cultured in a broth containing 20% horse serum (McLeod, 1912) the haemolysin that was produced, although being oxygen stable, was not antigenic (Todd, 1932a), whilst haemolysin produced by haemolytic streptococci cultured in a broth, which did not contain serum but was enriched with yeast extract (Neill and Mallory, 1926), was antigenic, although also oxygen labile. If the haemolysin produced from yeast extract broth was inactivated by incubation in air at 38°C for six hours it could be reactivated by reducing substances such as sodium hydrosulphite; more prolonged incubation caused irreversible oxidation of the haemolysin.

The increased quantity of haemolysin, produced by the stimulated growth of streptococci in a meat infusion broth with added glucose, was offset by increased destruction of the haemolysin by lactic acid produced from bacterial fermentation of the glucose (Hewitt, 1932a). The addition of sodium bicarbonate and inorganic phosphate buffered the acid. Furthermore, the inorganic phosphate was also shown to be essential for bacterial growth (Hewitt, 1932b).

The work culminated in the development of a meat extract broth that contained proteose peptone, dextrose, sodium bicarbonate, disodium hydrogen phosphate and sodium chloride (Todd and Hewitt, 1932). Todd Hewitt Broth has since become a general medium for the culture of streptococci as well as for the production of antigenic streptococcal haemolysin.

1.1.21b Tryptose Media

Kakavas, Palmer, Hay and Biddle (1942) added tryptose to a blood agar medium in order to enhance the cultivation of streptococci from milk samples incubated on the agar. Sharma and Packer (1970) used a tryptose broth, which also contained beef extract, yeast extract and proteose-peptone, to incubate swabs, prepared from bovine milk samples and skin sites, at an undisclosed temperature for 24 hours. Portions of the tryptose broth were then incubated on an agar containing inulin at 37°C for 48 hours. Potential *S. uberis* colonies were reincubated in the tryptose broth for between 18 and 24 hours before biochemical differentiation by subculture in broths containing various substrates.

1.1.21c Milk Enrichment Media

During studies into the transmission of *S. agalactiae* from cow to cow (Chodkowski and Lancaster, 1949), swabs of udder and teat skin, milking utensils and cowshed fittings were preincubated in either glucose broth, serum broth, autoclaved milk or autoclaved milk containing various dyes, before being subcultured on Edwards agar. When the level of faecal contamination was low and the concentration of faecal micro-organisms was of the same magnitude as that of the *S. agalactiae*, autoclaved milk was found to be the most selective medium, all added dyes having an inhibitory action on the *S. agalactiae*. However, when the level of faecal contamination was high, autoclaved

milk containing potassium tellurite (1:20,000) and boric acid (1:500) was the most selective medium for *S. agalactiae*. Although the crystal violet in the Edwards agar medium used in this work would have had some inhibitory action on non-streptococcal organisms, aesculin, the main differential substrate, would have been of no benefit to the growth of *S. agalactiae* as this species does not hydrolyse it (Buxton and Fraser, 1977; Rotta, 1986; Carter and Cole, 1990).

The milk, potassium tellurite and boric acid medium of Chodkowski and Lancaster (1949) was used to enhance the isolation of *S. uberis* from milk samples and swabs collected from various bovine skin sites (Cullen, 1966). Preincubation in the milk medium increased the isolation rate of *S. uberis* when the numbers of the bacteria were too low to be detected by direct incubation on agar plates. Cullen (1966) also noted that preincubation in heat treated milk with no additives produced a higher isolation rate of *S. uberis* than preincubation in beef infusion broth.

1.1.21d Litmus Milk

Twenty-six of 448 bovine skin swabs, which had been thoroughly shaken in two or four millilitres of "half strength Lemco broth", produced cultures of *S. uberis* when 0.1ml of each broth was incubated on inulin agar (Bramley *et al*, 1979). The remaining 422 swabs showed the presence of *S. uberis* only after one millilitre of each charged broth had been incubated in nine millilitres of litmus milk at 37° for 16 hours and an unrecorded volume of this mixture subcultured on the inulin agar. Similarly, *S. uberis* was recovered from only nine of 27 vulval swabs and ten of 21 rectal swabs by directly subculturing the broth samples on inulin agar; the remaining 29 cultures appeared only after incubating the broth in litmus milk before subculturing on inulin agar as above.

Only six of 65 isolates of *S. uberis* obtained from 715 bovine skin swabs, with veal infusion broth used as both wetting agent and transport medium, were obtained by direct inoculation of the broth on to aesculin agar (Buddle, Tagg and Ralston, 1988). The remaining 59 isolates only appeared after broth samples had been preincubated in litmus milk (0.3ml of veal infusion broth in 2.7ml litmus milk) at 37°C for 24 or 48 hours prior to subculture on the agar.

Streptococcus uberis was recovered from four of ten bovine vulval swabs shaken in half strength nutrient broth No. 2 (Oxoid Ltd., Wade Road, Basingstoke, Hampshire, RG24 0PW, England) by directly subculturing the charged broth on inulin agar (Kruze and Bramley, 1982); six further isolates were recovered only after one millilitre of each charged broth was incubated in nine millilitres of litmus milk at 37°C for 18 hours prior to being subcultured on the agar. By the same method 15 faecal swabs showed the presence of *S. uberis* by direct subculture of the charged broth on inulin agar, but no additional cultures of *S. uberis* were recovered using the litmus milk step. However, in another study, nine of 14 isolates of *S. uberis* were recovered from faecal samples mixed in the same nutrient broth only after initial incubation of the broth in litmus milk before subculture on inulin agar (Bramley, 1982). In none of these reports was the composition or concentration of the litmus in the milk detailed.

1.2 CHARACTERISATION OF AND VARIATION IN *STREPTOCOCCUS UBERIS*

1.2.1 BIOCHEMICAL PROPERTIES OF *STREPTOCOCCUS UBERIS*

Ayers and Mudge (1922) studied the characteristics of 100 cultures of streptococci isolated from 55 milk samples "from the udders of apparently normal cows. A few, however, were from cows having mastitis". The numbers of clinically infected and

apparently normal cows were not specified, and the only discrimination made was that larger numbers of bacterial colonies grew on the blood agar inoculated with milk from the clinical cases compared with milk from the apparently normal udders. Milk samples were collected in sterile tubes, "the first two or three streams being rejected". The tests used to differentiate the streptococci were haemolysis of blood agar (Brown, 1919), incubation in 0.01% methylene blue milk, fermentation of one per cent solutions of dextrose, inulin, lactose, mannite, raffinose, saccharose and salicin, as well as the production of carbon dioxide from dextrose, and ammonia from peptone (Ayers, Rupp and Mudge, 1921), and the hydrolysis of sodium hippurate (Ayres and Rupp, 1922); results of incubation in litmus milk at 10°C and 37°C were not referred to in the text but were recorded in the table. Seventy-nine of the 100 cultures, although differing in the type of haemolysis and in the fermentation of salicin, were grouped together as *S. mastitidis* because of their uniformity of reactions to all the fermentation tests except that of salicin (Ayers and Mudge, 1922). The *S. mastitidis* isolated from cows, with its ability to hydrolyse sodium hippurate and to ferment dextrose yeast broth to a limiting pH of 4.5, was shown to be a different species to *S. pyogenes*, isolated from man, which could not hydrolyse sodium hippurate and could only ferment dextrose yeast broth to a limiting pH of 5.5.

Of the other 21 cultures (Ayers and Mudge, 1922), three (their group E) were recorded as causing "gamma some green" haemolysis, reducing methylene blue milk (concentration 0.01%) in two to five days, acidifying litmus milk when incubated at 37°C but only slightly acidifying it when incubated at 10°C, hydrolysing sodium hippurate, and fermenting dextrose (limiting pH 4.6), inulin (pH 4.6), lactose (pH 4.5), mannite (pH 4.6), saccharose (pH 4.6) and salicin (pH 4.6), but not raffinose (pH 7.4) (appendix I). Although no aesculin test was performed, Cullen (1969) considered it possible that, in describing the three isolates of group E, Ayers and Mudge (1922) were the first to recognise the species that later became known as *Streptococcus uberis*,

however Cullen (1969) also included another two strains, Ayers and Mudge's group F, as having the characteristics of *S. uberis* even though these isolates did not ferment inulin. Ayers and Mudge (1922) grouped another 12 cultures together under the name *Streptococcus acidominus* on account of the small amount of acidity produced in all the fermentation reactions.

From cases of bovine mastitis or suspected mastitis, Klimmer and Haupt (1926) identified 44 strains of non-beta haemolytic streptococci as *S. agalactiae* Guillebeau, although three of the isolates fermented inulin (Minett *et al*, 1929). Two years later, from a total of 351 streptococcal isolates, Klimmer, Haupt and Roots (1928) described a number of strains (18 in text, 19 in table) of their group II (group I being *S. agalactiae*), which were later considered likely to be *S. uberis* by Seeley (1951) and Cullen (1969) although the original work did not mention whether the isolates could ferment inulin or hydrolyse aesculin. Cullen (1969) also included the other two strains of the Klimmer *et al* (1928) group II as being *S. uberis*, making a total of 20 (21), even though they only fermented lactose broth to a pH of 5.3, as opposed to a pH of 5.0 for the other 18 (19) isolates, and did not split sodium hippurate as completely as the others in the group. All the strains of the group II streptococci of Klimmer *et al* (1928) were reported to have come from a single cow affected with clinical mastitis (Minett *et al*, 1929).

Minett *et al* (1929) surveyed the types of bacteria found in milk samples collected from 113 cases of bovine mastitis from which 82 cultures of streptococci were recovered. Isolation procedures started on the day the samples were collected or the day after, however no details of overnight storage were given. Haemolytic and non-haemolytic streptococci were differentiated by the method of Brown (1919). Pure cultures were subcultured in serum broth, plain broth, litmus milk (incubated at 15° to 18°C, and at 37°C), one per cent glucose broth (acidity measured after five days), plain broth containing one per cent sodium hippurate (Ayres and Rupp, 1922), and fermentation

media using the same organic substrates as Ayers and Mudge (1922), which Brown (1937) noted "did not, unfortunately, include sorbitol or trehalose"; the sorbitol fermentation test differentiates *S. pyogenes*, *S. agalactiae*, *S. bovis* and *S. faecium* (non-sorbitol fermenters) from *S. uberis* and *S. faecalis* (sorbitol fermenters) with variable results being given by *Streptococcus dysgalactiae* (*S. dysgalactiae*) (Cowan and Steele, 1974). However, according to the API 20 STREP system of identification (bio Mérieux, 1990; bio Mérieux UK Ltd., Grafton House, Grafton Way, Basingstoke, Hampshire, RG22 6HY, England), sorbitol can no longer be included as a non-fermented substrate for *S. faecium* as a sub-species *S. faecium casseliflavus* can ferment sorbitol.

Fifty-two of the 82 streptococcal isolates (Minett *et al*, 1929) were described as haemolytic, that is beta haemolytic, and, despite not all the tests being applied to all 52 isolates, they were identified as being *Streptococcus mastitidis bovis* with the characteristics of acidifying, clotting and partially reducing litmus milk, fermenting glucose (32 of the 36 isolates tested having a limiting pH of less than 5.0, the other four being above 5.0), lactose (with the exception of one isolate), saccharose, and salicin (with the exception of six isolates), but not mannite, inulin or, for the 30 isolates tested, raffinose; 37 of the 42 isolates tested hydrolysed sodium hippurate. Of the 30 non-beta haemolytic isolates, 27 were of the "green-producing variety" and three were gamma haemolytic; the properties of 14 of these isolates (group I) conformed with those of the 52 (beta) haemolytic isolates with the exception of their lack of (beta) haemolysis and the other 16 isolates differed in their weaker clotting of litmus milk, diminished ability to ferment salicin and hydrolyse sodium hippurate and, of the 13 isolates tested, only two had a limiting pH of less than 5.0 in glucose broth. Since publication of the papers of Minett *et al* (1929) and Ayers and Mudge (1922), it has been found that the various strains of *S. agalactiae* (*S. mastitidis*) can show either alpha, beta or gamma haemolysis (Rotta, 1986). Minett *et al* (1929) also occasionally isolated

a small non-haemolytic streptococcus that fermented both inulin and mannite: In common with the report of Ayers and Mudge (1922), this was merely commented upon as there was "no satisfactory evidence that this type is associated with mastitis".

The cultural and biochemical characteristics of 107 strains of non-beta haemolytic streptococci isolated from visibly normal and abnormal milk samples were defined by Edwards (1932); non-beta haemolytic streptococci included the alpha and gamma types (Brown, 1919) together with colonies that "were surrounded by a green zone". The comprehensive set of tests included growth in litmus milk (no concentration given) at 10°C and 37°C, reduction of methylene blue milk (1:20,000), aesculin "fermentation" (Harrison and van der Leck, 1909a,b), hydrolysis of sodium hippurate (Ayers and Rupp, 1922), final hydrogen ion concentration in one per cent glucose broth (Avery and Cullen, 1919), fermentation of inulin, lactose, mannite, raffinose, saccharose and salicin, and resistance to temperatures of 50°C and 60°C for 30 minutes. From these tests, all the strains were categorised into the five streptococcal groups I, II, III, IV and *S. lactis*. The litmus milk test was found to be unsatisfactory for the purposes of differentiation because coagulation of cultures in groups II and III would only occur "if the medium had been seeded with a relatively large number of organisms, and in this case reduction followed. With smaller numbers there was acidification only" (Edwards, 1932).

The characteristics of group I streptococci (Edwards, 1932) corresponded with "the well recognised group of mastitis streptococci to which the name *S. agalactiae* has been applied", most of the isolates being cultured from milk samples collected from cows affected with "chronic catarrhal mastitis, the form of the disease with which *S. agalactiae* is now well recognised to be associated". Most of the group II streptococci were isolated from milk samples collected from acute udder infections that invariably culminated in permanent damage to the quarter; however a few cultures were isolated

from milk samples from infections in which the appearance of the milk rapidly returned to normal. The group II isolates differed from *S. agalactiae* in having a higher final pH in one per cent glucose broth (5.0 to 5.3 as opposed to 4.4 to 4.7) and in not being able to ferment salicin or hydrolyse sodium hippurate; neither group II or *S. agalactiae* hydrolysed aesculin. Group II isolates fermented lactose and saccharose and had a variable action on litmus milk ranging from slight acidification to clotting and reduction within 48 hours. In the same year, Diernhofer (1932) described a species of streptococcus, isolated from milk samples, that differed from non-haemolytic *S. agalactiae* in being able to reduce and acidify litmus milk without causing it to coagulate; *S. agalactiae* normally caused acidification and coagulation. Diernhofer named the new species *Streptococcus dysgalactiae.*

Five years later, Plastridge and Hartsell (1937) described a "new species" of streptococci which they named *Streptococcus pseudo-agalactiae* (*S. pseudo-agalactiae*). However, it was later realised that this species was the same as *S. dysgalactiae* (Diernhofer, 1932) and group II (Edwards, 1932) (Little, Brown and Plastridge, 1946). Plastridge and Hartsell (1937) reported that the fermentation reactions of *S. pseudo-agalactiae* (*S. dysgalactiae*) and its lack of ability to split aesculin mimicked *S. agalactiae*, but differentiated both species from the saprophytic streptococci. In addition, *S. pseudo-agalactiae* (*S. dysgalactiae*) differed from *S. agalactiae* in taking longer to acidify litmus milk (seven days as opposed to three) and in being able to reduce, even though only slightly, methylene blue milk. However, the major difference between the two species was in their serological properties, *S. agalactiae* being in group B of Lancefield (1933) whilst *S. pseudo-agalactiae* (*S. dysgalactiae*) was in group C. The two species also differed in their epidemiology, *S. agalactiae* infection being uncommon in cows in their first lactation but becoming an increasing problem with age, whilst the incidence of *S. pseudo-agalactiae* (*S. dysgalactiae*) infection was highest in first

lactation cows; however, infection with *S. pseudo-agalactiae* (*S. dysgalactiae*) was far less common than with *S. agalactiae* (Plastridge and Hartsell, 1937).

In describing the group III isolates, Edwards (1932) referred to previous work (Minett *et al*, 1929) in which streptococci had been isolated that were capable of fermenting inulin and mannite but "at that stage there was no satisfactory evidence that these (streptococci) were related to pathological changes in the udder". All 33 group III isolates fermented inulin and mannite, together with glucose (final pH 4.5 to 4.8), lactose, saccharose and salicin, but not raffinose; sodium hippurate was "invariably" hydrolysed, but there was no action on aesculin. Methylene blue milk (concentration 1:20,000) was reduced and "usually" coagulated and the action on litmus milk at 37°C varied from slight acidification without clotting to clotting and reduction, whilst at 10°C there was only slight acidification. Group III isolates also survived 30 minutes at a temperature of 50°C, but did not withstand a similar period at 60°C.

Edwards (1932) used the combined aesculin bile salt substrate (Harrison and van der Leck, 1909a,b) to test for aesculin "fermentation", despite being dubious as to the validity of this combined test, "it seemed probable that a positive result with the aesculin test depended firstly upon the ability of lactis strains to grow in the presence of bile salt, and secondly on the fermentation of aesculin". This hypothesis was tested in a separate experiment, reported later in the same paper, in which ten strains of each of the streptococcal groups I, II, III and *S. lactis* were subcultured into two aesculin mediums, one with no bile salt included, the other containing 0.25% sodium taurocholate. None of the streptococci from groups I, II or III grew in the combined aesculin and bile salt medium, and only the group III strains fermented aesculin in the absence of bile salt; *S. lactis* fermented the aesculin in both mediums "this being obviously due primarily to the ability to grow in the presence of bile salt". Although the contribution that the bile

salt made to the results of the aesculin test was realised, the earlier finding of the non-aesculin fermenting characteristic of the group III streptococci was not retracted.

In the same year as Edwards reported his group III streptococci, Diernhofer (1932) gave the name *Streptococcus uberis* to a species of bacteria, isolated from bovine milk, that hydrolysed aesculin and sodium hippurate, fermented glucose (limiting pH 4.7), inulin, lactose, mannite, saccharose and salicin, but not glycerin or raffinose, "temporarily" reduced methylene blue milk (no concentration given) without coagulation, and acidified litmus milk (at an unspecified temperature) without reduction or coagulation although, at 10°C, a sparse inoculum did not cause any acidification. According to Seeley (1951) and Cullen (1969), Diernhofer also received some of Edwards' group III strains and agreed that they were the same species as *S. uberis.*

Two years later, similar properties were reported of 14 isolates of the streptococcal group Ba (Plastridge, Anderson, Brigham and Spaulding, 1934). Milk samples were collected "aseptically....after discarding the first four streams". These isolates caused variable reduction of methylene blue milk (concentration 1:5,000) and "slight acidification to definite acidification, with coagulation and partial reduction" of litmus milk. Aesculin and sodium hippurate were hydrolysed and all strains fermented dextrose (final pH 4.6 to 5.0), galactose, levulose, maltose and sucrose, but not arabinose, dulcitol, glycerol, raffinose and xylose; in addition, "mannitol, salicin, sorbitol, trehalose and inulin, with one exception, were consistently fermented" although the exception was not clarified. In serological tests (Plastridge and Hartsell, 1937) "extracts of group Ba organisms failed to react with antiserums other than anti-group Ba serums". Plastridge and Williams (1939) subsequently acknowledged that the properties of group Ba were synonymous with those of *S. uberis* (Diernhofer, 1932).

The group IV streptococci (Edwards, 1932) were considered synonymous with *S. bovis* (Orla-Jensen, 1919; Ayers and Mudge, 1922), and the characteristics of the *S. lactis* isolates agreed with the description of the species given by Orla-Jensen (1919).

All 25 isolates of *S. uberis* reported by Ferguson (1938) produced a "green" haemolysis after incubation on blood agar at 37°C for up to 48 hours, and they also hydrolysed aesculin and sodium hippurate. No methylene blue milk test was reported and no acidification was noted in the litmus milk test, emphasis with the latter test being placed on whether reduction, when it occurred, happened before or after coagulation. All 25 isolates fermented mannitol and the 19 that were tested fermented amygdalin, inulin, salicin, sorbitol and trehalose, but not raffinose and xylose. Four of the 19 isolates were also tested with glucose and lactose, which were fermented, and with glycerol, which was not. The same four isolates also showed no growth at 10°C or at 50°C, but an unspecified number of them grew at 45°C. No explanation was given as to why the additional tests were carried out on only a proportion of the isolates.

Plastridge and Williams (1939) identified 141 isolates of *S. uberis* by gamma or "weakly beta" (presumably alpha) haemolysis of blood agar, acidification of litmus milk with slight or no reduction, a variable response to methylene blue milk (concentration 1:5,000), and hydrolysis of aesculin. Sodium hippurate was "usually" hydrolysed, and lactose, mannitol, sorbitol, trehalose and "usually" inulin were fermented, but not arabinose and raffinose. One hundred and thirty-six of the isolates were placed in 11 serological groups by slide agglutination tests of which about 20% showing a common antigen with more than one serological type; the other five isolates failed to react with any of the type sera employed.

From a total of 680 cultures of "weakly haemolytic" streptococci, 83 isolates were identified as *S. uberis* (Slanetz and Naghski, 1940) in that they reduced methylene blue

milk (concentration 1:20,000), gave variable reactions to litmus milk (acidification only, acidification and reduction, or acidification, reduction and clotting), hydrolysed aesculin, and fermented mannitol and salicin, but not raffinose. However, 15 of the isolates failed to hydrolyse sodium hippurate and nine did not ferment inulin; whether any isolate failed both these tests was not stated. Seventy of the isolates were tested for their ability to grow at various temperatures; ten isolates grew at 10°C and 60 isolates grew at 45°C, but none survived 30 minutes incubation at 60°C. In serological tests, seven of the 17 isolates tested by the precipitin method and 12 of the 36 isolates tested by agglutination were positive to two antisera collected from rabbits that had been inoculated with cultures of groups Ba and Ba_2 streptococci of Plastridge and Hartsell (1937).

The 14 cultures of *S. uberis* described by Miller and Heishman (1940) were recovered from milk samples collected immediately before a regular milking without discarding the first stream of milk, but after the udder had been washed and the teat end wiped with a "pledget" of cotton moistened with disinfectant solution consisting of "one part of bichloride of mercury to 1,000 parts of 50 per cent alcohol". The isolates produced no haemolysis on blood agar, but acidified litmus milk with variations in reduction and coagulation, and showed a variable response to methylene blue milk (concentration 1:10,000). All isolates hydrolysed aesculin and sodium hippurate, and fermented inulin, lactose (final pH 4.5), mannite, salicin and sorbitol, but not arabinose and raffinose, and an undefined number did not ferment trehalose.

Little (1940) studied 24 isolates of *S. uberis* and identified the bacterium by its "green" haemolysis of blood agar, coagulation and reduction of methylene blue milk (concentration 1:5,000), hydrolysis of aesculin and sodium hippurate, and fermentation of dextrose (final pH 4.6 to 4.9), inulin, lactose, maltose, mannite, saccharose, salicin, sorbitol and trehalose. The viability of the isolates was destroyed by a temperature of

60°C for 30 minutes, and one strain grew sparingly on 40% bile agar. The author noted reports of atypical properties of some strains of *S. uberis*, hippurate negative strains (Slanetz and Naghski, 1940) and inulin negative strains (Slanetz and Naghski, 1940), but found that the latter could not be included as *S. uberis* as they possessed other characteristics untypical of the species.

Francis (1941) isolated eight strains of "group III mastitis streptococci (Edwards, 1932)" from seven pairs of bovine tonsils. The type of haemolysis was "studied" but not divulged. All eight isolates reduced, and some coagulated, methylene blue milk (concentration 1:15,000), and all isolates acidified, coagulated and reduced litmus milk. Aesculin and sodium hippurate were hydrolysed, and glucose (final pH 4.5 to 4.9), inulin, lactose and mannite, but not raffinose, were fermented. Three of the isolates hydrolysed starch and five grew at 45°C, although it was not stated whether both properties were seen in the same isolate; none of the eight isolates were viable after 30 minutes at 60°C. In a description of a further eight isolates that were reported to resemble the group III streptococci, one isolate failed to ferment inulin, another four failed to hydrolyse sodium hippurate, and the other three fermented raffinose.

Little *et al* (1946) stated that isolates of *S. uberis* "usually" produced alpha or gamma haemolysis of blood agar, reduced methylene blue milk (concentration 1:20,000), "fermented" aesculin, hydrolysed sodium hippurate, produced ammonia from arginine, and fermented inulin, lactose, mannitol, salicin, sorbitol and trehalose, but not arabinose and raffinose. Other properties, which appeared in tabulated form, showed that *S. uberis* also acidified and coagulated litmus milk with partial or no reduction, caused a variable reaction to methylene blue milk (concentration 1:5,000), and fermented glucose (final pH 4.6 to 5.0), maltose and sucrose. The same authors also reported unpublished data by Plastridge and Williams that placed "277 biochemically similar strains" into 15 serological groups by precipitin and agglutination tests. About

one third of these isolates cross-agglutinated with up to five different antisera, with a wider range of "cross-reactions" by the precipitin method. About ten per cent of these isolates did not ferment inulin, although they were found to be serologically identical with some of the inulin positive isolates.

The 52 isolates of *S. uberis* reported by Seeley (1951) produced an "indifferent or slight greening" haemolysis of blood agar, and acidified litmus milk with about half the isolates also reducing and coagulating it; one isolate showed "resistance to 0.1% methylene blue". All isolates hydrolysed aesculin and sodium hippurate, although "about ten per cent" hydrolysed sodium hippurate "very weakly", this variability being thought to be a trait of the species, and 11 isolates hydrolysed starch. Fructose, glucose (final pH 4.6 to 4.9), lactose, maltose, mannitol, salicin, sorbitol, sucrose and trehalose were fermented by all the isolates, however nine isolates did not ferment inulin and one isolate fermented raffinose; whether the latter was also one of the inulin negative isolates was not stated. None of the isolates fermented arabinose and xylose but all slowly fermented glycerol under aerobic but not anaerobic conditions, the latter test being considered a means of distinguishing *S. uberis* from *S. lactis*, which did not ferment glycerol, and from *S. faecalis*, which fermented glycerol both aerobically and anaerobically. None of the 11 isolates tested fermented melibiose and only one of 11 isolates tested fermented dulcitol. All 11 isolates tested tolerated a ten per cent concentration of bile salts and five of these tolerated a 40% concentration. Of the 52 isolates, 51 grew at 10°C, 50 grew at 45°C and 42 were found to be viable after 30 minutes at 60°C. The author found that the ability of *S. uberis* to grow at various extremes of temperature depended on the previous treatment of the isolate; isolates that grew well at 45°C lost the ability to grow at that temperature after being stored at 4°C for several weeks, whilst isolates grew well at 45°C after being stored at room temperature for several months. Typing *S. uberis* in terms of the serological, physiological and nutritional characteristics was found to be futile as the reactions of

S. uberis were not comparable with the properties of any one streptococcal group or division.

Colonies causing browning of a medium that the author called crystal violet aesculin thallium acetate blood agar, the formulation of which was not recorded, were subjected to four further biochemical tests (Hughes, 1960). Colonies grown on this medium that fermented inulin and mannite, but not raffinose, and hydrolysed sodium hippurate were accepted as being *S. uberis*. Isolates "occasionally gave positive precipitin tests with Lancefield's group D or E antisera".

Sweeney (1964) isolated potential *S. uberis* organisms from bovine orifices using dry swabs, and from bovine skin using swabs moistened with "infusion broth" (no further details given). Each charged swab was agitated in five millilitres of infusion broth after which 0.1ml of the broth was incubated on aesculin crystal violet agar (Edwards, 1933) at an undefined temperature for 48 hours. Aesculin hydrolysing isolates were subjected to undefined biochemical tests, which yielded a total of 96 isolates of *S. uberis*. Milk samples from the same cows were similarly incubated on five per cent blood agar and "suspicious" colonies subcultured on the Edwards agar; 18 isolates of *S. uberis* were identified by the undefined biochemical tests. When tested against Lancefield group E antiserum, 21% (the actual number was not recorded) of the *S. uberis* isolates collected from the udder skin and 16% of the isolates originating from the milk were Lancefield group E positive.

The cows used by Sweeney (1964) were sampled in their following lactation by Cullen (1966), samples being collected from the skin, orifices and milk by the same methods. Milk samples were collected before normal milking, after the udders had been cleaned with water and individual paper towels; no disinfectant udder washes were used. Broth samples were incubated on both blood agar and aesculin agar (Edwards, 1933) at 37°C

for 48 hours, and 0.1ml of broth was also incubated "overnight" in five millilitres of Chodkowski medium (Chodkowski and Lancaster, 1949) before being incubated on the aesculin agar. Milk samples were similarly directly incubated on the agars and also, interpreting an ambiguous text, after first being incubated overnight either on their own or in Chodkowski medium. Four hundred and eighty-six isolates were identified as *S. uberis* in that they did not reduce methylene blue milk (concentration 1:1000), but caused "slight acidity to acid clotting and reduction in litmus milk", hydrolysed aesculin and sodium hippurate, and fermented inulin, mannitol, salicin, sorbitol, sucrose and trehalose, but not raffinose; 83 isolates (17%) "reacted" with Lancefield group E serum. Another 89 isolates "with accepted variations in pattern" were included as being *S. uberis* due to the fact that a proportion of these isolates were found to be Lancefield group E positive, 21 of the isolates being sorbitol negative of which 13 (62%) were group E positive, and the remaining 68 isolates being raffinose positive of which four (six per cent) were group E positive.

In the following year, a definitive biochemical classification of *S. uberis* was attempted (Cullen, 1967) because "the serological classification of *S. uberis* has not yet been fully elucidated and there has been much disagreement on the biochemical properties of the organism" as demonstrated by reference to inulin and sodium hippurate negative strains (Slanetz and Naghski, 1940), trehalose negative strains (Miller and Heishman, 1940), inulin negative strains (Little *et al*, 1946), and inulin negative, raffinose positive and methylene blue positive strains (Seeley, 1951). A total of 1,481 isolates of aesculin hydrolysing streptococci were recovered from milk samples collected from clinical cases of mastitis and subclinical infections discovered at routine herd sampling (the criteria for such infections was not defined), and also from various skin sites and orifices. The normal reactions of *S. uberis* were defined as either alpha or gamma haemolysis of blood agar, darkening of the area under the colonies growing on blood aesculin agar, no action on methylene blue milk (1:1,000), acidification of litmus milk with clotting and

slight reduction which, later in the same paper, was revised to a variable response from acidification alone to acidification with clotting and reduction, hydrolysis of aesculin and sodium hippurate with hydrolysis of starch being a "variable feature", production of ammonia from arginine, and acid production from inulin, lactose, mannitol, salicin, sorbitol and trehalose, but not raffinose. Colonies growing on blood aesculin agar that were surrounded by wide rings of darkened medium were found never to be biochemically similar to *S. uberis*, and colonies having the appearance of *S. uberis* would often be *S. bovis* or some other species. Thirty-eight (21%) of the 184 isolates collected from the clinical cases of mastitis that conformed to the above parameters were Lancefield group E positive using the precipitin test, with 11 (ten per cent) and 83 (18%) group E positive for the 105 isolates from subclinical cases and 456 skin isolates respectively. Isolates that conformed with the above definition of *S. uberis* in every way with the exception of either fermenting raffinose or not fermenting sorbitol were also included as *S. uberis* as a proportion of them were found to be Lancefield group E positive. Of the raffinose positive isolates, four (28%) of 14 from clinical cases, one (17%) of six from subclinical cases and four (six per cent) of 68 from skin samples were group E positive, as were three (33%) of nine from clinical cases, nought (nought per cent) of two from subclinical cases, and 13 (62%) of 21 from skin samples of the sorbitol negative isolates respectively. Isolates that had the uncharacteristic feature of being litmus milk or inulin negative, or methylene blue positive were recorded as not being *S. uberis* as they usually showed other untypical features and never gave a positive Lancefield group E reaction. The author stated that, based on biochemical tests, there was a closer relationship between *S. uberis* and *S. faecalis*, the latter having the properties of not fermenting inulin and belonging to Lancefield group D (Carter and Cole, 1990), than between *S. uberis* and *Streptococcus infrequens* (*S. infrequens*), even though *S. infrequens* was the other major species of streptococcus that belonged in Lancefield group E. *Streptococcus infrequens* had already been shown to be beta

haemolytic, occasionally to coagulate litmus milk after prolonged incubation, and not to ferment inulin or hydrolyse sodium hippurate (Deibel, Yao, Jacobs and Niven, 1964).

Various bovine skin sites were sampled using swabs moistened with tryptose broth (Sharma and Packer, 1970), and milk samples were collected after the udders had been washed with warm water, dried, and the teat tips cleaned with a "pledget" of cotton soaked in ethyl alcohol. Both skin swabs and milk samples were incubated in tryptose broth at 37°C for 24 hours after which portions of broth were subcultured on a medium that was described as "Edwards medium (modified)", however it contained no aesculin, the main differential constituent of Edwards medium, but did contain sodium azide (0.5g per l) and a small quantity of inulin (1.0g per l), neither of which is normally included in Edwards medium. Inulin fermenting colonies (how these were differentiated was not explained) were incubated in tryptose broth for 18 to 24 hours before being subjected to fermentation tests. One hundred and seventy-two isolates that hydrolysed sodium hippurate and fermented inulin, mannite, salicin and sorbitol, but not raffinose, were classified as *S. uberis*. The precedent for identifying *S. uberis* using a non-aesculin Edwards medium had been set some years earlier when inulin, mannite and sorbitol had been substituted for aesculin "which war conditions had made impossible to purchase" (Seeley, Anderson and Plastridge, 1945).

A wide range of substrates were used to identify 173 bovine and two ovine isolates of what were credited as being *S. uberis* (Roguinsky, 1971), although not all the differentiating tests were performed on all the isolates. All 160 isolates tested for "growth" in methylene blue milk (concentration 0.005%) gave a positive result, as did 49 out of 50 isolates in an 0.01% concentration and two of 160 isolates in an 0.1% concentration. All 175 isolates hydrolysed aesculin and, with one exception in each case, sodium hippurate and arginine. In fermentation tests using all 175 isolates, mannitol and trehalose were the only substrates to be fermented by all 175 isolates,

whilst 158 isolates fermented inulin, 172 sorbitol, 12 raffinose and one arabinose. All ten isolates tested on cellobiose, maltose, mannose and salicin fermented the substrates, whilst none of the 20 isolates tested fermented xylose and none of the 30 isolates tested fermented glycerol aerobically although the time allowed for this was not reported, and an anaerobic fermentation test was not performed. All 160 isolates tested grew at 10°C and nine of the 175 isolates grew at 45°C. In serological tests, 76 (43%) of the 175 isolates could not be identified with any of the Lancefield groups, 65 (37%) were Lancefield group E positive, 18 (ten per cent) group P, eight (five per cent) group G and three isolates were group U; four isolates belonged to group P as well as to one other group, and one isolate was classified into group B, the group for *S. agalactiae.* Fourteen of the 17 inulin negative isolates could not be placed in any Lancefield group, and of the other three isolates, two were group E and one group P. Of the 12 raffinose positive isolates, two could not be placed in any Lancefield group, three were group E, four group G and three group P.

Misra and Marshall (1972) described two strains of *S. uberis* that appeared as large grey mucoid smooth segmented colonies after incubation on blood agar at 37°C for 48 hours. The mucoid property was lost when the strains were repeatedly alternately subcultured in trypticase soy agar containing 0.1% Tween 80 and on blood agar. Both mucoid and non-mucoid variants of the two isolates tolerated an 0.01% but not an 0.1% concentration of methylene blue milk, hydrolysed aesculin, arginine and sodium hippurate, and fermented cellobiose, glucose (final pH for the mucoid variants being 5.3 and 5.1, and the non-mucoid variants 4.7 and 4.8 respectively), inulin, lactose, maltose, mannose, salicin, sorbitol and trehalose, but not arabinose, raffinose or xylose. However, both isolates displayed weak beta haemolysis and survived a temperature of 62.8°C for 30 minutes with a five log decrease in numbers. The two strains differed in that one grew in the presence of a four per cent concentration of sodium chloride, did not ferment melezitose, but reduced litmus milk when incubated between 21° and 55°C, acidifying

it between 21° and 45°C, and causing it to clot at 45°C, whilst the other isolate only tolerated a two per cent concentration of sodium chloride, fermented melezitose but only acidified litmus milk when incubated between 32° and 37°C. The percentage of guanine and cytosine in the deoxyribonucleic acid (DNA) of the two isolates was found to be 38.4 and 39.3.

By analysis of the percentage of guanine and cytosine in the DNA of 31 isolates of *S. uberis* (Garvie and Bramley, 1979), all but two of the isolates could be classified into two main genotypes called group I and group II. All 20 isolates in group I acidified litmus milk to some degree with some slightly reducing it, hydrolysed aesculin, arginine and sodium hippurate, and fermented mannitol, salicin, sorbitol and trehalose; one isolate did not ferment inulin and three fermented raffinose. All but one of the group I isolates, including the four biochemically unusual isolates, had a percentage of guanine and cytosine of between 36.2 and 37.0; the other biochemically conventional group I isolate had a percentage of guanine and cytosine of 35.5, which put it in the middle of the range of 34.8 to 35.9% guanine and cytosine of the nine isolates of group II. The nine group II isolates were biochemically less uniform with only two isolates showing all the properties of the 16 conventional isolates in group I; of the seven others, three fermented raffinose, one did not ferment inulin, one did not ferment inulin and sorbitol, one fermented both inulin and sorbitol only slightly and was not able to hydrolyse sodium hippurate, and one did not hydrolyse sodium hippurate. The authors maintained that the two isolates that could not be classified into either group I or group II might also have been *S. uberis* even though both were unable to ferment mannitol and sorbitol and, in addition, one was unable to hydrolyse aesculin or ferment inulin, whilst the other could not hydrolyse sodium hippurate and could ferment inulin only slightly.

Swab samples were collected from various bovine skin sites, vulvas and rectums of cows in two herds on different farms, together with swab samples of the sand-bedded cubicle

floors of one of the farms and the straw yards of the other farm. The swabs were suspended in either two or four millilitres (depending on the size of the swab) of "half strength Lemco broth" before 0.1ml of each charged broth was incubated on inulin crystal violet agar (Pugh and Bramley, 1977) at 37°C for 40 hours (Bramley *et al*, 1979); one millilitre of each charged broth was also incubated with nine millilitres of litmus milk at 37°C for 16 hours before being subcultured on the agar. Potential *S. uberis* colonies from the inulin crystal violet agar were subcultured on calf blood agar containing 0.1% aesculin at 37°C for 48 hours before inoculation into inulin and raffinose broths (Garvie and Bramley, 1979), and acid citrate broth (Gibson and Abdel-Malek, 1945). Isolates that produced acid from inulin but not raffinose, and did not grow in, or produce gas from, acid citrate broth were recorded as being *S. uberis*. The identity of ten per cent of these isolates was further confirmed by a more extensive range of physiological tests (Garvie and Bramley, 1979). Only nine (4.8%) of 186 swab broths from the cows of one herd and 34 (11%) of 309 swab broths from the cows of the other herd yielded *S. uberis* isolates without preincubation in litmus milk. Only three milk samples from one of the herds yielded isolates of *S. uberis*; however it was reported that the milk isolation procedures were those used by Bramley (1976) who, in that paper, had reported a method of isolating *Escherichia coli* (*E. coli*) from milk samples collected from udders previously infused with that pathogen. All eight (32%) of the 25 swab samples of the cubicle sand that yielded *S. uberis* did so only after enrichment in litmus milk, and of the 36 swab samples of the straw bedding, 17 (47%) were positive for *S. uberis*, four of the swabs yielding *S. uberis* from direct inoculation of the agar medium.

Bovine faecal samples were plated on inulin agar both directly and after preincubation in litmus milk at 37°C for 18 hours (Bramley, 1982), and ten gram samples of straw bedding were treated with 100ml "half strength nutrient broth" in a "stomacher" (no further details given) for one minute after which tenfold dilutions were subcultured on

inulin agar and incubated at 37°C for up to 48 hours. Inulin fermenting colonies were subcultured on aesculin blood agar (Higgs and Bramley, 1981). Aesculin hydrolysing streptococci were identified as *S. uberis* if they could ferment inulin (for a second time) and produce ammonia from the hydrolysis of arginine (Niven, Smiley and Sherman, 1942), but were not able to produce carbon dioxide from acid citrate (Gibson and Abdel-Malek, 1945) or ferment raffinose. The isolates with these properties were then confirmed as *S. uberis* by further biochemical tests as detailed by Garvie and Bramley (1979). Fourteen isolates of *S. uberis* were recovered from faecal samples but, for nine of the isolates, this was only possible following enrichment of the samples in litmus milk.

In screening bovine rectal and vulval swabs for isolates of *S. uberis* (Kruze and Bramley, 1982), the charged swabs were first agitated in "half strength nutrient broth" and 0.1ml of each broth was incubated on inulin agar (Bramley, 1982) at 37°C for 48 hours; in addition, one millilitre of the charged broth was incubated in nine millilitres of litmus milk at 37°C for 18 hours before being subcultured on inulin agar. Inulin fermenting colonies were subcultured on aesculin blood agar and isolates were identified as *S. uberis* by further biochemical tests (Bramley, 1982). All 15 isolates of *S. uberis* recovered from rectal swabs and four of the ten isolates recovered from vulval swabs appeared without the use of litmus milk as an enrichment medium, whilst isolates from the remaining six vulval swabs appeared only with inclusion of the litmus milk step.

Robinson, Jackson and Marr (1983; 1985) collected milk samples after discarding the foremilk and thoroughly cleaning the teat end with cotton wool soaked in methanol. The authors were satisfied that *S. uberis* could be recovered and identified by incubating the milk samples on an undefined aesculin nutrient blood agar at 37°C for 48 hours after which identification of potential *S. uberis* isolates was confirmed by "inulin and raffinose sugar reactions".

Bramley (1984) stated "the probability that an aesculin hydrolysing streptococcus isolated from bovine mastitis is *S. uberis* is high", and of 1,894 aesculin hydrolysing streptococcal isolates from cases of bovine mastitis examined by the National Institute for Research in Dairying, 82.3% (no specific numbers were recorded) were *S. uberis*, 8.2% *S. faecium* and 4.9% *S. faecalis*. The typical biochemical reactions of *S. uberis* were given as gamma or slight alpha haemolysis of blood agar, hydrolysis of aesculin and arginine, but not starch or gelatin, production of acid from inulin, mannitol, salicin, sorbitol and trehalose, but not raffinose, and growth at 10°C with some growth at 45°C. However, the author stated that a 99% accurate identification of *S. uberis* could be made with just four tests, fermentation of inulin, but not raffinose, hydrolysis of arginine to release ammonia, and an inability to grow and produce gas in acid citrate broth. The other one per cent included untypical *S. uberis* that were able to ferment raffinose or could not ferment inulin or hydrolyse arginine.

Aungier and Austin (1987) incubated milk samples on blood agar at 37°C for 48 hours, after which a tentative identification of bacterial isolates was made on the basis of colony morphology, agar haemolysis and Gram staining. Streptococci were further identified by biochemical tests that included aesculin and sodium hippurate hydrolysis and the fermentation of inulin, sorbitol and trehalose, however the individual properties of the streptococcal species recorded were not defined.

Buddle *et al* (1988) collected bovine milk samples and swab samples from rectums and various skin sites. The swabs were moistened with "veal infusion broth" and, after sampling, the charged swabs were vortexed in 1.8ml broth after which 40μl portions were incubated on both "crystal violet aesculin blood agar" and "aesculin blood agar" at 37°C for 48 hours; 0.3ml of charged broth was also incubated in 2.7ml litmus milk at 37°C for up to 48 hours before being subcultured on aesculin blood agar. Milk samples were collected prior to the udder being washed but after the teat ends had been swabbed

with 70% alcohol and the first ten to 15ml of milk discarded. The milk samples were incubated on aesculin blood agar at 37°C for 48 hours and aesculin fermenting colonies that produced acid when inoculated into inulin and sorbitol broths, but did not ferment raffinose, were recorded as *S. uberis*. The identity of 20 of the 70 isolates recovered was confirmed by the use of API 20 STREP kits, or by ensuring that the isolates could hydrolyse sodium hippurate and ferment both salicin and mannitol. Only six of 70 isolates were recovered without initial enrichment in litmus milk.

Gram positive cocci that hydrolysed aesculin were subjected to five further tests (Hill and Leigh, 1989). Isolates that hydrolysed sodium hippurate, produced ammonia from arginine, fermented inulin but not raffinose, and could not neutralise or produce gas from acid citrate were identified as *S. uberis.*

1.2.2 TYPING ISOLATES OF *STREPTOCOCCUS UBERIS*

A method of typing isolates of *S. uberis*, which utilised the bacterium's ability to produce various bacteriocin-like inhibitory substances (P-typing) and its sensitivity to similar substances produced by other bacteria (S-typing), was proposed by Tagg and Vugler (1986). The biochemical and physiological tests used to identify the isolates as *S. uberis* were not recorded. An isolate to be typed was first plated as a streak culture on Columbia agar base medium (Gibco Laboratories, Madison, WI, USA) supplemented with 5% (w/v) human blood and 0.5% (w/v) calcium carbonate, and incubated anaerobically at 37°C for 18 hours. After all growth had been removed and the surface of the agar sterilised with chloroform, nine indicator cultures (eight streptococcal isolates of various Lancefield groups and one isolate of *Micrococcus luteus*) were inoculated across the original streak and the plate reincubated. The pattern of growth or inhibition of the indicator cultures was recorded as the isolate's P-type. A reverse of the above procedure was carried out for the sensitivity tests (S-type) in that six streptococcal

indicator strains of various Lancefield groups were initially streaked on the agar. Following incubation, removal of the bacterial growth and sterilisation of the agar surface, the isolate of *S. uberis* was inoculated across the streaks. The pattern of growth or inhibition of growth of the *S. uberis* isolate was recorded as its S-type. Of the 15 isolates of *S. uberis* tested, 12 produced inhibitor substances by P-typing with nine different patterns detected. All 15 isolates were able to be typed by S-typing, ten different patterns being distinguished. By combining both P-type and S-type results, all 15 isolates had an inimitable pattern of inhibitory characteristics.

Isolates of *S. uberis* have also been typed according to the restriction fragment size of chromosomal DNA remaining after digestion by the endonuclease *Hin*d III (Hill and Leigh, 1989). The restriction fragments of DNA were separated by gel electrophoresis, the discriminating region of the fragment length being between nine and 23 kilobases (kb). By subjecting a photograph of the tracks of restriction fragments to an image analysing densitometric scan, which plotted intensity against migration, computer aided analysis of the patterns of the fragments could be made.

Jayarao, Oliver, Tagg and Matthews (1991) compared the bacteriocin-like inhibition method of typing (Tagg and Vugler, 1986) with that of the restriction endonuclease method (Hill and Leigh, 1989) using forty-two isolates of *S. uberis*; however 15 of the isolates had one or more atypical reactions to arginine, sodium hippurate, inulin, lactose, raffinose or ribose. In addition, all isolates were serotyped using A, B, C, D, F and G group specific antibodies and four were found to belong to group G; serotyping for group E, to which about 20% of *S. uberis* isolates belong (Cullen, 1967) was not recorded. The 42 isolates produced 12 different bacteriocin-like inhibitory patterns and 17 restriction endonuclease patterns. A close association was found between the restriction endonuclease patterns and the bacteriocin-like inhibitory patterns.

More recently, strains of *S. uberis* have been typed by the technique of DNA amplification fingerprinting, in which one or more short oligonucleotide primers of arbitrary sequence were used to produce a characteristic spectrum of amplified DNA fragments (Jayarao, Bassam, Caetano-Anollés, Gresshoff and Oliver, 1992). In comparison with the restriction endonuclease typing system, DNA amplification fingerprinting grouped 22 strains of *S. uberis* into 15 patterns whilst the restriction endonuclease fingerprinting grouped them into 12 patterns. One specific DNA amplification primer known as 8.6d (5'GTAACGCC3') was particularly effective.

1.2.3 DIFFERENTIATION OF THE SPECIES INTO *STREPTOCOCCUS UBERIS* AND *STREPTOCOCCUS PARAUBERIS*

The genotypes I and II of *S. uberis* found by Garvie and Bramley (1979) were further distinguished by determination of the difference in the nucleotide sequences of the 16S ribosomal-RNA (r-RNA) of both genotypes (Williams and Collins, 1990) using a dideoxynucleotide reverse transcriptase method (Lane, Pace, Olsen, Stahl, Sogin and Pace, 1985). The 16S r-RNA of both genotypes was shown to be composed of 1,480 nucleotides and the nucleotide sequence of both genotypes had about 96% homology, which was comparable with the degree of homology of existing separate streptococcal species. The authors stated that the two genotypes differed at 36 nucleotide sites and, as strains within a single species generally possessed far fewer nucleotide differences, the two genotypes were phylogenetically different. The genotype II was given the name *Streptococcus parauberis* (*S. parauberis*). The biochemical properties of *S. parauberis* were very similar to genotype I (still called *S. uberis*) except for *S. parauberis* having a varying ability to hydrolyse sodium hippurate. A year later, *Hin*d III digest DNA restriction patterns showed differences between the genotypes of *S. uberis* and *S. parauberis* and considerable variation between different strains within each genotype

(Williams and Collins, 1991). Similar results were obtained using four restriction endonucleases (Jayarao, Doré, Baumbach, Matthews and Oliver, 1991).

Groschup, Hahn and Timoney (1991) found that both *S. uberis* and *S. parauberis* had identical biochemical properties although *S. parauberis* showed variation in its ability to utilise sodium hippurate, inulin, mannitol, raffinose, ribose and sorbitol; the only consistent cultural characteristic by which *S. parauberis* could be distinguished from *S. uberis* was in the ability of *S. parauberis* to grow at 10°C.

1.3 SURVEYS OF *STREPTOCOCCUS UBERIS* INFECTION

Following the isolation and identification of *S. uberis* (Diernhofer, 1932; Edwards, 1932), the species began to be included in the list of pathogens found in surveys of bovine mastitis although, for some time, its importance was not recognised. In one of the first surveys to include *S. uberis*, Ferguson (1938) examined 2620 foremilk samples, from 655 cows in 18 herds, for the presence of streptococci; it was not recorded whether any of the cows were showing signs of clinical mastitis. Streptococci were recovered from 229 quarter milk samples (8.7%) from 138 cows (21%) of which 160 (70% of the infected samples) were *S. agalactiae*, 25 (10.8%) *S. uberis*, 16 (seven per cent) *S. dysgalactiae* and 28 unidentified streptococci. The 25 milk samples from which *S. uberis* was isolated came from cows in seven of the herds.

Two years later, Slanetz and Naghski (1940) isolated 680 strains of weakly haemolytic streptococci from an unrecorded number of milk samples collected from an unrecorded number of cows in ten herds showing evidence of streptococcal mastitis infection, the criteria for streptococcal infection being "when streptococci and 500,000 or more leukocytes per cc were present in incubated milk samples". Five hundred and seventy-three isolates (84.3%) were identified as *S. agalactiae*, 83 (12.2%) as

S. uberis of which 15 did not hydrolyse sodium hippurate and nine did not ferment inulin, 15 (2.2%) as *S. dysgalactiae*, and the remaining nine (1.3%) as *S. faecalis*. In an addendum, the authors reported that they had isolated *S. uberis* from the udder tissues of two of five freshly slaughtered cows; no details were given of how the isolates were recovered from the tissues. All five cows were presumed to have been free of mastitis in their last two or three lactations, the presumption being based on tests carried out on milk samples collected every three months, with a final milk sample being collected just before the cows were slaughtered; tests on the milk samples included incubation on blood agar, and microscopic examination for leucocytes and streptococci.

A survey of what, in the absence of any statement to the contrary, must be presumed to have been milk samples from 1,200 clinical cases of mastitis was carried out in 1942 by the Ministry of Agriculture, Fisheries and Food (MAFF) Central Veterinary Laboratory (CVL) at Weybridge, England (Wilson, 1963). Although the actual numbers of cases from which each pathogen was isolated was not recorded, *S. agalactiae* was isolated from 44%, *S. dysgalactiae* from 10 per cent, and *S. uberis* from four per cent of the cases; staphylococci were isolated from 25% of the cases (*Staph. pyogenes* from 17% and non-haemolytic staphylococci from eight per cent). The protocol for identification of the various pathogens was not recorded.

Twenty-five herds were reported to be cleared of infection with *S. agalactiae* by "blitz" treatment with penicillin in 1947 (Wilson, 1954). No details were given as to how many cows had been culled and how many replacements had entered the herds in the intervening period but, when the herds were re-examined four years later in 1951, 11 cases of *S. agalactiae* infection were found compared with 338 at the time of the treatment and, during the same period, the number of *S. dysgalactiae* infections fell from 58 to 40. However, *Staph. pyogenes* infections rose from 81 to 86, and those of

S. uberis more than doubled from 52 in 1947 to 117 in 1951. The protocol for identification of the various pathogens was not recorded.

In another survey carried out by the CVL, 2,005 samples (once more it has to be presumed milk samples) from cases of clinical mastitis received by the CVL at Weybridge in 1952 (Stableforth, 1953), *Staph. pyogenes* was isolated from 18% of the samples (no actual numbers of samples were recorded), *S. uberis* from 12%, *S. agalactiae* from nine per cent and *S. dysgalactiae* from eight per cent of the samples; no pathogen could be isolated from 27% of the samples, and the remaining samples contained various other unidentified organisms. The protocol for identification of the various pathogens was not recorded. It was thought that the high proportion of negative samples was due to animals having been treated before being sampled, and the low proportion of cases due to *S. agalactiae* was a reflection of the successful use of penicillin in the treatment of mastitis caused by this pathogen.

In the five years from 1949 to 1953 inclusive, milk samples from all clinical cases of bovine mastitis seen in one veterinary practice were examined for bacterial pathogens (Hughes, 1960). The practice had a dairy population of approximately 4,000 cows and heifers, and during the five years a total of 2,684 milk samples were collected. Mastitis infections in 1949 totalled 625; this rose to 654 infections in the next year and then decreased steadily in the following three years to 308 infections in 1953. Over the five years, *S. uberis* was isolated from 484 (18.0%) of all pretreatment milk samples, the incidence decreasing from 116 (18.5%) in 1949 to 56 (18.2%) in 1953, with a range in the intervening years of between 16.2 and 19.8%. Only *S. agalactiae* had a higher total of infections, 689 (25.6%), over the five years with an incidence rate of 180 (28.8%) in 1949 declining to 63 (20.4%) in 1953. Over the same five year period *Staphylococcus aureus* (*Staph. aureus*) and *S. dysgalactiae* infections totalled 337 (12.5%) and 302 (11.2%) respectively.

All 141 dairy herds with more than 20 cows per herd that were within a ten mile radius of Reading, England were visited during 1958 and 1959 (Howell, Wilson and Vessey, 1964) and information recorded on the type of milking machine used and its operation, and any clinical abnormalities of the 4,929 udders that were examined; a "bulk" milk sample from all functioning quarters of each cow was also collected. Of the 2267 infected milk samples recovered (46% of the total number of samples analysed), only 17 were found from which *Staph. pyogenes*, *S. agalactiae*, *S. dysgalactiae* or *S. uberis* could not be isolated either singly or in combination. The protocol for identification of the various pathogens was not recorded. The actual number of milk samples from which each pathogen was isolated and the number of herds and cows affected with each pathogen was not reported, but *S. uberis* on its own was isolated from 7.5% of the milk samples and, as one of a mixed culture with one or more of the other three organisms, in another 2.9% of the milk samples, giving 10.4% in total; seasonal variations were reported as being insignificant. Of the other three pathogens, *Staph. pyogenes* was isolated as a pure culture in 17.9% of the milk samples and in another 5.5% of samples as one of a mixed culture, with *S. agalactiae* isolated from 8.7% and 2.9%, and *S. dysgalactiae* from 4.5% and 2.0% of the milk samples respectively. Of the 141 herds in the survey, 83% were infected with *S. uberis* with an average of 12% of cows infected in each affected herd. *Streptococcus agalactiae* was found in 49% of the herds (24% of cows infected per herd), *S. dysgalactiae* in 58% of the herds (ten per cent) and *Staph. pyogenes* in 95% of the herds (24%). No range or breakdown of the different infections was given.

Another survey, presumably of milk samples, from 3,800 cases of mastitis examined at the CVL at Weybridge in 1962 (Wilson, 1963) showed that *S. agalactiae* accounted for four per cent, *S. dysgalactiae* eight per cent, and *S. uberis* ten per cent of infections with *Staph. pyogenes* and non-haemolytic staphylococci causing a further 39% of infections; actual numbers of cases from which each species was isolated, and the protocol for

identification of the various pathogens, were not recorded. As *S. agalactiae* was always susceptible to penicillin, the author thought it probable that many cases of *S. agalactiae* were being successfully treated, with only those that remained refractory being sampled for bacterial isolation; because of this the percentage of mastitis cases caused by *S. agalactiae* would, in reality, be higher than the four per cent that had been found.

The prevalence of subclinical mastitis was investigated by the examination of 500 herds throughout Great Britain (England, Scotland and Wales), selected by size and geographical location from the agricultural census returns for June 1975 (Wilson and Richards, 1980). Each herd was assigned a month when it was to be sampled so that the investigations in each size group and in each geographical region were evenly distributed; all the herds were visited once during the 12 month period of the survey to collect quarter milk samples. Every cow that was in milk was sampled unless it had calved within one week of the visit, and individual quarters receiving antibacterial therapy were not sampled. With herds of more than 100 milking cows, the sampling was restricted to 100 animals although it was not recorded as to how the 100 cows were chosen. In the course of the survey 27,526 cows in 501 herds were sampled. The milk samples were delivered to a MAFF Veterinary Investigation Centre the same day, or kept at 5°C for delivery the following morning. *Staphylococcus pyogenes* was isolated from 8,790 (8.1%) of the quarter milk samples, these coming from 5,689 (20.7%) cows in 467 (93.2%) of the herds, *S. agalactiae* was isolated from 3,694 (3.4%) of samples from 1,992 (7.2%) cows in 197 (39.3%) herds, *S. uberis* was isolated from 1,610 (1.5%) of samples from 1,267 (4.6%) cows in 369 (73.7%) of herds, and *S. dysgalactiae* was isolated from 1,195 (1.1%) of samples from 968 (3.5%) cows in 311 (62.1%) of herds. Of the other pathogens, *E. coli* was isolated from less than 0.1% of all quarters. The protocol for identification of the various pathogens was not recorded. A wide variation in the prevalence of the four major types of infection was seen amongst the regions. The highest prevalence of milk samples infected with *S. uberis* came from the two regions of

Southern England, and Devon and Cornwall, with 2.5% and 2.7% of infected quarter samples respectively; Scotland had the lowest prevalence of *S. uberis* with only 0.9% of samples infected. The overall prevalence of udder infections was found to decline as herd size increased although no breakdown of figures for each type of infection was given. In herds of between ten and 20 cows, the proportion of quarters infected with a major pathogen was 21.2%, whilst in herds of more than 100 cows the figure fell to 11.9%. This could be explained by the significant correlation in herd size and the adoption of hygiene control measures; only 26.9% of farms with between ten and 20 cows used teat dips and dry cow therapy, this percentage rising as the herd size increased with 76.3% of farms with over 100 cows using the hygiene control measures.

Wilesmith, Francis and Wilson (1986) conducted a detailed survey from 1980 to 1982 inclusive to determine the incidence of clinical mastitis and associated bacteria in 45,000 cows from 378 herds throughout England and Wales; the herds were selected to obtain as much variation as possible with regard to teat dipping and dry cow therapy, although no relationship between the level of mastitis control practices and mastitis incidence was attempted. Actual numbers of clinical cases associated with each pathogen and the protocol for identification of the various pathogens were not recorded. *Staphylococcus aureus*, *S. agalactiae* and *S. dysgalactiae* had incidence rates that ranged from 14.8 to 16.5%, 1.3 to 1.8% and 8.6 to 9.7% *per annum* respectively. The incidence of *S. uberis* rose from 13.4% in 1980 to 16.9% in 1982, and it was the predominant pathogen isolated in the summer (May to September) of 1982. During the period of the survey, *S. uberis* was the most frequently isolated pathogen from clinical mastitis in non-lactating cows with an incidence of 15.9 to 16.6%; the authors reported that in the 1983, this figure rose to 23.0%. *Escherichia coli* was the most common pathogen encountered over the three years of the survey with an incidence that ranged from 17.6 to 19.4% *per annum.*

The importance of bovine mastitis as an economic loss to the dairy industry prompted an investigation into the incidence and nature of mastitis in a small number of herds (Aungier and Austin, 1987). In an initial survey of 681 lactating cows in seven herds in 1983, 22 cases of clinical mastitis (3.2% of cows) were found of which 12 were due to *S. uberis*, two to *Staph. aureus* and eight to *E. coli*; neither *S. agalactiae* or *S. dysgalactiae* were recovered. However the survey revealed a high prevalence of subclinical infections, *S. uberis* being recovered from over 19% of all lactating quarters "and *Staph. aureus* from a further 7%"; these infections "persisted for months" without showing clinical signs. A total of 533 cases of clinical mastitis were investigated both in the initial survey and on five of the farms (two farms took no further part after the initial survey) over the following six months; this represented 11.5 cases per 100 cows per month. *Streptococcus uberis* was isolated in 89 (17%) of the cases, and was second only to *Staph. aureus* with 97 (18%) of cases. *Escherichia coli* was responsible for 65 (12%) and *S. dysgalactiae* for 14 (three per cent) of the cases; there was a single case from which *S. agalactiae* was isolated.

From milk samples submitted for examination to the Scottish Agricultural Colleges' Veterinary Investigation Service between 1979 and 1989, between 50 and 100 isolates of *S. uberis* were identified annually, with the exception of 1983 when there were 130 isolates (Scottish Veterinary Investigation Service, 1989). However in the same period, whilst isolations of most other mastitis pathogens declined, the percentage of isolates of *S. uberis*, as a total of all mastitis pathogens, rose from five per cent in 1979 to 16% in 1989.

A more recent survey (Logue, Gunn and Fenlon, 1993) reported that the rates of isolation of different mastitis causing organisms throughout Great Britain over the previous decade had remained remarkably consistent. However, from 1980 to 1992, the Scottish Veterinary Investigation Service had noted a slight increase in the isolation

of *Staph. aureus*, *S. agalactiae* and *S. dysgalactiae* from milk samples sent to the service compared with that of the whole of Great Britain, whilst *S. uberis* was isolated from approximately 10% of Scottish milk samples (by interpretation of a bar chart), which was far lower than the 17% recorded for the whole of Great Britain. From both Scotland and the whole of Great Britain, the proportion of milk samples from which *E. coli* was isolated was almost identical at around 25%.

Booth (1993) noted that the incidence of clinical mastitis in England and Wales over the previous 30 years had fallen from 153 cases per 100 cows *per annum* in 1962-63 to 34 cases per 100 cows *per annum* in 1986-87 and, allowing for inconsistences of surveys, the current incidence was likely to be around 40 cases per 100 cows *per annum*. However, the annual incidence of both *S. uberis* and coliform mastitis remained at about seven cases per 100 cows, thus the proportion of cases of mastitis caused by these two pathogens had increased markedly, so much so that, over the last 15 years, about a quarter of the milk samples processed by the laboratories of the Veterinary Investigation Service showed infection with *E. coli* whilst infections due to *S. uberis* increased over the same period from 13.4% to 18%, and those due to coagulase positive staphylococci rose from 12.7% to 17.1%, all other pathogens each being diagnosed in less than ten per cent of cases. Booth (1993) also noted the reduction in prevalence of subclinical mastitis caused by *Staph. aureus* and the three major streptococcal species over the ten years from 1966/67 to 1977; as the same author had noted previously (Booth, 1988), due to the difficulties of carrying out surveys into subclinical mastitis, no recent data was available.

1.4 EPIDEMIOLOGY OF *STREPTOCOCCUS UBERIS*

In contrast to *S. agalactiae*, which could only exist in the mammary gland and therefore, with rigorous hygiene measures, could be eliminated from a herd of cows (Minett,

Stableforth and Edwards, 1933), *S. uberis* was found to be able to survive on the exterior of the cow (King, 1981) and live in the environment (Cullen and Little, 1968, 1969; Bramley, 1982). This capacity for extra-mammary existence has been considered to be the major factor in the epidemiology of *S. uberis* infection, and in the inability of hygiene measures and antibacterial therapy to control it (Bramley, 1984).

In a survey of 100 pairs of bovine tonsils, eight strains of group III streptococci (Edwards, 1932) were isolated from seven of the pairs (Francis, 1941), and in four cases, they appeared to be the predominant organism. Another eight strains differed in one of three biochemical characteristics of *S. uberis* as described by Cullen (1967). Swabs of vaginal secretions were also collected from 40 dairy cows, from which one strain of group III streptococci (Edwards, 1932) was isolated, however its biochemical characteristics were not stated.

During the spring of 1943, Neave, Sloan and Mattick (1944) investigated an outbreak of clinical mastitis in a dairy herd, thought to be caused by a faulty vacuum gauge. Every three months in the three years prior to the clinical outbreak, the milk from each cow had been examined for bacteria but the findings were not disclosed. No clinical mastitis had occurred in the previous six months and there had been only two cases in the previous year (1942), one caused by *S. uberis* and the other by a haemolytic staphylococcus. Milk from the majority of the infected quarters was found to contain "large numbers" of *S. uberis*; the protocol for identification of the various pathogens was not recorded. Without any explanation as to how it had been detected, the authors stated that "this organism (*S. uberis*) was known to be on the teats of the cows before the outbreak, as early as February 1942".

Crossman, Dodd, Lee and Neave (1950) monitored a cow that developed subclinical *S. uberis* infection in one quarter of her udder during the dry period and which persisted

until the end of the following lactation. Although no external signs of clinical disease were present, the yield and percentage solids-not-fat of the quarter remained well below the performance of the three uninfected quarters.

From a study of the "great majority" of cases of bovine clinical mastitis seen in a veterinary practice in Cheshire, England (Berger and Francis, 1951), a seasonal difference in the incidence of mastitis caused by different bacterial agents was noted, *S. uberis* being categorised as one of the organisms chiefly associated with winter mastitis. Although no description of winter housing was given, the total incidence of mastitis of all types was found to be highest during the third quarter of the year "when the cows have been out at grass all summer, and before they are brought into the cowshed". The protocol for identification of the various pathogens was not recorded.

Hughes (1960) reported that the incidence of mastitis caused by *S. uberis* was higher in the winter months (November to April) than during the rest of the year, the data being collected from all the clinical cases of mastitis attended by the veterinary surgeons of one practice over several years. No details of the housing and management of the cows was recorded. In comparing incidence of infection with stage of lactation, *S. uberis* was found to be similar to the other common mastitis pathogens in that infection was highest during early lactation (two to seven weeks post-partum); however, there were appreciable numbers of infections from which *S. uberis* was isolated throughout the rest of the lactation and in the dry period. A cow's susceptibility to mastitis infection increased during the first four lactations after which it declined; this phenomenon was attributed to the fact that the more mastitis susceptible cows were culled at an early age, thus leaving the more resistant animals in the herd.

Following the work of Neave *et al* (1944) who had found *S. uberis* on teat skin, and Slot (1958) who isolated four strains of *S. uberis* from 119 samples of cow and calf

faeces, an epidemiological survey of *S. uberis* was undertaken in a herd of 24 dairy cows to ascertain the extent to which the pathogen could live outside the mammary gland (Sweeney, 1964). Sampling started in late November and continued for nine months. Milk samples were collected from each lactating quarter once a week and pretreatment samples were taken from all four quarters in cases of clinical mastitis. Every fortnight, swab samples were collected from various sites on the cows' bodies, these being the left and right sides of the udder, teat surfaces, lips, nostrils, inner canthi of the eyes, posterior parts of both vagina and rectum, poll, chest wall, sacrum, caudal folds and belly. *Streptococcus uberis* was isolated from 18 out of a total of 990 milk samples, from 70 out of a total of 492 udder surface swab samples, and from 26 out of a total of 5,412 swab samples from the other sites. The majority of the isolations of *S. uberis* from all sources were collected in the period from the end of November to the beginning of May. The udder skin was found to be the commonest site from which *S. uberis* was isolated and milk infection was always coincident with the presence of the pathogen on the udder surface indicating to the author that infections of the mammary gland parenchyma were secondary to that of the udder skin.

During the following year Cullen (1966), working with the same herd of 24 cows as Sweeney (1964), carried out a similar survey although the extra-mammary sampling sites were reduced to both sides of the udder wall, tips of the teats, lips, vagina, rectum, sacrum and ventral midline. The survey started in late January and continued to the middle of the following December. All but six of the 24 cows calved in January or February, with the remainder calving in the following four months. *Streptococcus uberis* was isolated frequently during the year, with the highest number of isolations being recorded in the month of May. Over the sampling period, 574 isolates of *S. uberis* were collected and the pathogen was recovered at least five times from all the sites sampled and between six and 47 times from each cow. A total of 170 isolates were collected from both sides of the udder, 148 from the belly skin, and 91 from the

lips; all other sites, including the tip of the teat, gave much lower numbers of isolates. For a period of four weeks in August and September, the lips were the only site from which *S. uberis* could be isolated, which suggested the possibility that this area could act as a reservoir for the organism, as well as a means of spreading the infection. However the low incidence of isolations from just five rectal swabs indicated that passage of *S. uberis* through the whole length of the alimentary tract was not common, and that faecal contamination was unlikely to be important in the spread of the infection. *Streptococcus uberis* was not isolated from milk samples collected from the end of June until the beginning of October, and from the udder skin and tips of the teats from the middle of August until the beginning of October. With the more or less constant colonisation of *S. uberis* on the lips and ventral abdomen for the greater part of the year, the author concluded that infection of the milk was secondary to that of the skin, and that total eradication of the pathogen from a herd would be difficult.

By passing a length of nylon tubing into the rumen through an incision in the left sub-lumbar fossa, samples of ruminal fluid were collected from 18 of a herd of 24 cows "known to be infected with" *S. uberis* (Cullen and Little, 1968; 1969). "An organism resembling *S. uberis* biochemically" was found in the ruminal fluid of five of the cows, the biochemical tests described by Cullen (1967) being used to identify the isolates. The pathogen was also isolated from one rectal swab and seven lip swabs collected from the cows, and found in soil samples taken from the field being grazed by the cows, especially from the wetter areas of the field where the cows tended to congregate. It was assumed that the cows had originally infected the soil and, due to the reservoirs of *S. uberis* available in the environment and on the cows, it was likely that a herd, once infected with *S. uberis*, would remain infected and that clinical cases of *S. uberis* mastitis would continue to occur. No isolates of *S. uberis* were found in soil samples collected from a field grazed by a non-infected herd.

Sharma and Packer (1970) monitored the occurrence of *S. uberis* in a herd of 24 cows over one year, swab samples being collected weekly from udder skin, teat skin, lips, nostrils, vulva, rectum, dorsal neck, lateral thorax, sacral region, caudal folds and abdominal wall, as well as milk samples. Eighteen isolates of *S. uberis* were recovered from 568 milk samples, and 154 isolates were recovered from 6,836 swabs samples. The skin of the udder and teat was most commonly infected with nearly 50% of all skin isolates being recovered from these sites, and infections were present at these sites at the time that 16 of the 18 milk isolations occurred. *Streptococcus uberis* infection was found to be more prevalent in the winter months and in older cows, and occurred most often in early lactation.

Weekly milk samples, together with swab samples from the four teat apices, lips, shoulder, belly, flanks, escutcheon, vulva and rectum were collected from five cows in each of two herds for six weeks during the months of January and February (Bramley *et al*, 1979). Each herd was wintered under a different housing system and bedding samples were also collected. One herd of 80 cows, housed in cubicles bedded with sand, had a history of infrequent cases of clinical mastitis caused by *S. uberis* with just seven quarters (2.2%) in seven cows (8.8%) being infected in the previous 12 months. During the trial, *S. uberis* was never isolated from the milk samples of the five cows chosen, but it was isolated from 186 (51.7%) skin swabs, although the numbers of *S. uberis* colony forming units was always less than 600 from each swab. Only nine swabs showed the presence of *S. uberis* without the isolation procedure including initial enrichment in litmus milk. The shoulders, flank and escutcheon were the commonest sites of recovery, with the vulva and rectum being least common. The other herd of 44 cows, housed in strawed yards, had a history of 25 quarters (14.2%) from 13 cows (29.5%) being clinically infected with *S. uberis* in the previous year. The five cows sampled from this herd all calved during the sampling period. Examination of milk samples collected within 24 hours of calving showed that one quarter in each of three

cows was infected with *S. uberis*; results of the examination of milk samples collected subsequently were not recorded. The pathogen was isolated from 309 (85%) swab samples collected from this herd, with a higher proportion (11%) being recovered without enrichment in litmus milk. The distribution of the organism between the various swab sites was similar to that of the cubicle housed herd. The authors concluded that none of the sites sampled were a primary source of *S. uberis* for the dairy herds. Eight (32%) of the 25 swab samples of the cubicle sand yielded *S. uberis* but only after enrichment in litmus milk, whereas 17 (47%) of the 36 swab samples of the straw bedding were positive for *S. uberis*, four of the swabs yielding *S. uberis* from direct inoculation of the agar medium; colony numbers were low from both types of bedding.

During an outbreak of *S. uberis* mastitis on one farm, the bacterium was isolated from 19 out of 22 samples of straw bedding and from 14 out of 41 samples of fresh faeces (Bramley, 1982), although nine of the latter samples had to be enriched in litmus milk before yielding the pathogen. From another 32 herds, randomly selected with no known incidence of *S. uberis* mastitis, but all using straw as bedding, *S. uberis* was isolated from 31 out of 129 samples of bedding, all the isolates coming from 18 of the herds.

Swab samples from the vulvas and rectums of 14 dairy cows were collected at approximately weekly intervals during the months of April, May and June (Kruze and Bramley, 1982). Fifteen of the 98 rectal swabs (15.3%) and ten of the 98 vulval swabs (10.2%) were positive for *S. uberis*, most isolates being recovered without the use of enrichment medium; 16 of the 25 positive samples were from only two cows with the other isolates spread between the remaining 12 cows.

In a survey of about 660 cows in five herds monitored over four years, *S. uberis* was found to be the only major pathogen frequently isolated in large numbers from the teat ends of dry cows (Robinson *et al*, 1985). The authors also recorded a high incidence of dry period clinical mastitis from which *S. uberis* was isolated in the months of July, August and September: As there was a concurrent low incidence of dry period 'summer' mastitis associated with *Corynebacterium pyogenes* (now renamed *Actinomyces pyogenes*), the fly *Hydrotoea irritans* was considered unlikely to be involved in the spread of *S. uberis*. Although it had been proposed that bacterial colonisation of teat ends was significant in dry cow infections (Cousins, Higgs, Jackson, Neave and Dodd, 1980), only 20% of the quarters whose teat ends became infected with *S. uberis* during the dry period were found to have intramammary infection at calving (Robinson *et al*, 1985).

Over an eight month period, Buddle *et al* (1988) isolated *S. uberis* from 65 (9.1%) out of a total of 715 skin swabs taken from the abdominal wall, the medial aspect of the thigh and the teats of 11 cows. Only five (1.2%) isolates of *S. uberis* were recovered from 420 milk and dry period secretion samples and never from rectal swabs gathered from the same cows. Using the bacteriocin-like inhibition typing method of Tagg and Vugler (1986), numerous stains of *S. uberis* were recorded. The isolates of *S. uberis* recovered from samples simultaneously collected from the teat (whether from milk samples or skin swabs was not stated) and adjacent udder skin were found to have different type profiles, however in one case, isolates of *S. uberis* recovered from the teat skin and from the milk sample of the same quarter had identical profiles. Several isolates of *S. uberis*, all with different profiles, could be recovered from the same skin sample. *Streptococcus uberis* was isolated more frequently from both skin and milk samples collected during the winter months than during the spring and autumn; the cows were kept on pasture throughout the year.

Ten strains of *S. uberis* originally isolated from clinical cases of bovine mastitis were found to have differing susceptibility to being killed by the same concentration of blood polymorphonuclear leucocytes (neutrophils) (Hill, 1988). Two strains that represented the extreme opposite responses to this test were strain 0140J of which 32.4% survived after two hours incubation at 37°C in the presence of the neutrophils, and strain EF20 of which only 0.3% of survived the same treatment. When both strains were infused into separate quarters of 18 lactating cows and ten pregnant non-lactating cows, strain 0140J produced clinical disease in 16 of the 18 lactating quarters, whilst EF20 produced infection in just two quarters. Both strains caused infection in six of the ten non-lactating quarters, five of the dry cows being susceptible to both infections. Although the virulence of the two strains had been measured by their susceptibility to being killed by neutrophils in milk *in vitro*, the author noted that the two lactating cows that resisted infection by strain 0140J, and most of those that resisted infection by strain EF20, did so without the development of an apparent inflammatory response.

1.5 DEFENCE MECHANISMS OF THE BOVINE UDDER

1.5.1 THE TEAT ORIFICE

Local natural defence mechanisms in the bovine teat and udder are constantly in operation to prevent infection (Reiter and Bramley, 1975). The teat duct is the first barrier against pathogens gaining access to the interior of the udder, and cows with a small teat duct, although in consequence slow milkers, are less prone to mastitis infection than cows with larger teat ducts (Dodd and Neave, 1951). This finding was later confirmed by radiographic studies of teat ducts deliberately exposed to *Aerobacter aerogenes*, *S. agalactiae* and *Staph. aureus* several times a week for six and 13 weeks during lactation and, in other cows, for the first three weeks of the dry period (McDonald, 1975). In all three groups the average distal, middle and proximal teat

canal diameters of the non-infected teats were between 42 and 79% less than those of infected teats, and the average length of the non-infected teats was slightly shorter (four to six per cent) than the infected teats. It was thought that a longer teat duct might allow greater retention of bacteria within the canal.

The teat duct is formed from an invagination of the teat skin, which is highly keratinised (Hibbitt and Cole, 1968). The structure of the duct provides a mechanical defence system, which together with the flushing out of the duct during milking, reduces the risk of bacteria entering the teat (Reiter and Bramley, 1975). The longitudinal folds on the internal wall of the teat cistern terminate distally at Fürstenberg's rosette, the junction with the proximal end of the teat duct (Nickerson and Pankey, 1983). Contraction of the smooth muscle around the teat duct causing centripetal pressure on the loose folds of tissue at Fürstenberg's rosette was thought by some authors (Pounden and Grossman, 1950) to maintain closure of the proximal opening of the teat duct. However, other workers found no interlocking of the folds at Fürstenberg's rosette (Nickerson and Pankey, 1983), and that teats were capable of retaining milk even when all nerves to the teat had been excised by the drastic step of removing the udder from the cow (Peterson, 1944).

In the lactating cow the udder is protected from invading pathogens by the flushing effect of milk passing through the teat duct (Reiter and Bramley, 1975), although this physical defence mechanism is insufficient to prevent infections by *Staphylococcus* species persisting in the teat canal (Forbes and Herbert, 1968). Unlike the Gram positive Micrococcaceae, Gram negative species such as *E. coli* and *Klebsiella pneumoniae* have little ability to colonise and penetrate the duct of the lactating udder (Reiter and Bramley, 1975).

1.5.2 THE ROLE OF KERATIN

The integrity of the keratin layer lining the teat duct was shown to have an important role in preventing the entry of bacteria (Murphy, 1959). Removal of the keratin temporarily destroyed the barrier function in teats previously resistant to assault by a test strain of *S. agalactiae* but, following successful treatment of the infection by antibiotics, the keratin layer was reformed within four weeks of the injury and the teat returned to a resistant state.

Electron microscope studies showed that the keratinised cells bordering the lumen of the teat duct were fenestrated, with the spaces between the cells giving rise to a mesh-like structure, which appeared to impede the progress of bacteria (Chandler, Lepper and Wilcox, 1969). The structure of the teat duct keratin of quarters infected with clinical mastitis was similar to that of uninfected teats, with the exception of pus and debris being associated with the top layer of keratin lining the duct of the infected quarters.

McDonald (1970) examined two teats from each of two cows whose milk had been examined by the California mastitis test (Schalm and Noorlander, 1957) at every milking, and examined for the presence of bacteria at least once every three weeks, throughout their lives. The two teat ducts from quarters of one cow that had not shown signs of intramammary infection throughout five lactations and intervening dry periods were found to have a maximum diameter of 0.40 to 0.55mm, which decreased towards the distal end of the duct. The compact and dense keratin lining the duct was 0.09 to 0.40mm thick, firmly adherent to the epithelial surface and formed a small stellate-shaped opening with three to five potential channels for milk flow. In contrast, two teat ducts from the quarters of the other cow, which had succumbed to a total of 22 separate infections (11 coliform, seven *Staphylococcus epidermidis* and four *Staph. aureus*) during its four lactations and intervening dry periods, were of larger diameter (1.0 to

1.25mm) with the proximal part widely dilated and irregular. The mesh-like structure of the keratin was less dense than that of the other cow's teat ducts and only 0.02 to 0.12mm thick; in some areas keratin was absent altogether. A single large continuous opening for milk flow was present.

Keratinisation and desquamation of the stratum granulosum progressed in a distal direction towards the teat orifice (Nickerson and Pankey, 1983) and thus the greater thickness and mesh-like structure of the keratin lining was most effective at or near the meatus. However, narrow fissures in the keratinised surface orientated longitudinally to the duct were also present which, it was postulated, might support capillary movement of bacteria.

Whole teat duct keratin lacked antibacterial activity *in vitro* (Forbes, 1970); *Staph. aureus* was still viable after 27 days when incubated on teat duct keratin, and some sub-groups of the bacterium survived for 60 days. This supported previous work which had shown that teat canals could be infected for up to 18 weeks before the bacteria reached the teat sinus (Forbes and Herbert, 1968).

1.5.3 THE ROLE OF THE CATIONIC (BASIC) PROTEINS IN KERATIN

Antimicrobial properties of the cationic (basic) proteins and fatty acids of teat duct keratin *in vivo* remain unproven (Craven and Williams, 1985; Hill, 1992). Cationic proteins extracted from bovine teat duct keratin were shown to be able to cause a marked inhibition of growth on three mastitis-causing organisms, two phage types of *Staph. aureus* and one strain of *S. agalactiae* (Hibbitt and Cole, 1968; Hibbitt, Cole and Reiter, 1969). A 50% inhibition of growth occurred when 100 colony forming units (cfu) of each of the two strains of *Staph. aureus* were incubated in one millilitre of 0.01 molar (M) citrate buffer which contained two to five micrograms of cationic protein.

The strain of *S. agalactiae*, however, proved to be a little more resistant and required a concentration of 11.5μg per ml of cationic protein to produce the same level of inhibition. When the cationic proteins were preincubated with anionic polymers (DNA or heparin) the inhibiting effect of the proteins was completely destroyed, suggesting that attachment to the bacterial cell wall was by an electrovalent link (Hibbitt and Cole, 1968): Such a linkage was shown by use of the anionic dye Fast Green FCF, which only outlined staphylococci previously incubated with cationic proteins that were presumed to be bound to the bacterial cell surface.

Electron microscope studies showed that, when incubated at 37°C with a particular phage type of *Staph. aureus*, cationic proteins bound to the bacterial cell surface causing morphological changes and the loss of a discrete cell wall (MacMillan and Hibbitt, 1969); the staphylococcal cells also appeared to be surrounded by a layer of fibrillar material.

Staphylococcus aureus that had been incubated *in vitro* with teat duct cationic protein bound over six times more ^{131}iodine labelled bovine serum albumin than untreated control staphylococci (Hibbitt and Benians, 1971). After recovering the same phage type of *Staph. aureus* from the teat duct of cows where it had been deposited for 24 hours, it showed an even greater degree of binding to the labelled serum albumin, the ratio of albumin bound to the recovered bacteria compared to that bound to the control bacteria exceeding 22:1. Further work showed that when teat duct cationic proteins were incubated with *Staph. aureus* that had been previously cultured in a medium containing ^{14}carbon (^{14}C), there was a rapid release of ^{14}C labeled protoplasmic protein from the staphylococci followed by a slower release over the next five hours. The authors speculated that the action of the teat duct cationic proteins would not only provide the host with an initial natural line of defence against invading bacterial

pathogens but would also render the bacterial cell membrane more susceptible to the action of immunoglobulins.

In a living teat canal, the continuously synthesised supply of cationic proteins would bind to any negatively charged molecule present, including invading micro-organisms (Hibbitt *et al*, 1969). However, the bactericidal properties of cationic proteins were rapidly abolished *in vitro* by binding to the acidic milk protein casein (Reiter and Bramley, 1975), and thus any residues of milk that remained in the teat duct would be likely to reduce the bactericidal properties of the keratin protein.

1.5.4 THE ROLE OF LIPIDS IN KERATIN

Reports on the antimicrobial role of the lipid fraction of teat duct keratin have been conflicting (Hill, 1992). Adams (1962) was reported to have harvested over 100mg of keratin per teat duct from three fortnightly collections from each duct (Treece, Morse and Levy, 1966). Analysis of the material showed it to be composed mainly of lipid (90%). In subsequent *in vitro* studies, *S. agalactiae* was found to be most susceptible to the myristic (14-carbon) esterified (saturated) fatty acid fraction, and to the palmitoleic (16-carbon) and linoleic (18-carbon) non-esterified (unsaturated) fatty acid fractions (Adams and Rickard, 1963). The authors considered that the term 'keratin' should be replaced by 'lactosebum' to signify the sebum-like content of the material and that it originated from the lactiferous duct.

The amount of lipid in keratin and its role in natural resistance to bacterial invasion of the teat canal were later disputed (Treece *et al*, 1966). By pooling the harvest of up to 16 monthly collections, less than 20mg of keratin were obtained from each duct; the lipid content of this keratin averaged 36% and no relationship could be found between

the fatty acid composition and resistance to mastitis. These workers stated that the name keratin accurately described the very insoluble and proteinaceous nature of the material.

1.5.5 THE NATURAL BACTERIOSTATIC FACTORS IN FRESH MILK - LACTENINS

According to available evidence, Hesse (1894) was the first to observe that raw milk had an ability to inhibit many species of bacteria (Reiter, Pickering, Oram and Pope, 1963). When *S. pyogenes*, the streptococcal species responsible for causing scarlet fever, was inoculated into raw milk or milk heated to a temperature of 58°C for 20 minutes, multiplication of the bacterium was arrested and it eventually died (Jones, 1928). The same procedure carried out on "mastitis streptococci" caused only temporary inhibition, the bacteria growing rapidly after a lag period of six to eight hours; this was attributed to the pathogens becoming adapted to an "inhibitory agent" (Jones and Simms, 1929). The possibility of a strain of mastitis streptococci, resistant to the inhibitory agent, being present at the time of inoculation and multiplying unhindered was rejected on the basis that, if it had multiplied at the rate shown to be typical of the surviving forms, the lag phase would have been less than three hours. Both the scarlet fever streptococci and the mastitis streptococci grew readily in milk that had been boiled for five minutes and then cooled.

A year later, the same workers isolated the inhibitory agent from whey proteins and named it lactenin (Jones and Simms, 1930). More recently, lactenin has been found to be composed of several non-immunoglobulin factors including lactoferrin, lactoperoxidase and thiocyanate, the latter two being components of the lactoperoxidase, thiocyanate, hydrogen peroxide system (Reiter and Bramley, 1975).

1.5.6 LACTOFERRIN

The protein lactoferrin is a powerful chelator of iron, and is thus able to inhibit the growth of iron dependent micro-organisms *in vitro*, particularly Gram negative organisms such as *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and some Gram positive organisms such as *Staph. aureus* (Schanbacher and Smith, 1975): Streptococci have a low iron requirement (Reiter and Bramley, 1975).

Lactoferrin is found in all secretions (Reiter and Bramley, 1975). In the presence of bicarbonate it binds free iron thus inhibiting the multiplication of bacteria that have a high iron requirement; this process is counteracted by citrate, which is present in milk and colostrum and which competes with the lactoferrin for iron and makes the latter available to the bacteria. During the dry period, the decreased citrate and increased bicarbonate levels of the udder secretions allow lactoferrin to bind the available iron; iron-dependent pathogens such as *E. coli* that enter the udder during this period are, therefore, rarely able to proliferate. At parturition, the raised citrate and lowered bicarbonate levels of the colostrum and milk increase the amount of available iron, which leads to an increased susceptibility to coliform infection in the udder at or soon after parturition.

1.5.7 THE LACTOPEROXIDASE, THIOCYANATE, HYDROGEN PEROXIDE SYSTEM

Following work that showed one of the components of lactenin to be a peroxidase enzyme (Wright and Tramer, 1958; Stadhouders and Veringa, 1962), the growth in milk of some strains of *Streptococcus cremoris* (*S. cremoris*) was found to be inhibited by lactoperoxidase, providing thiocyanate ions (SCN^-) and hydrogen peroxide (H_2O_2)

were also present, the latter being produced under aerobic conditions by the organism being inhibited (Reiter *et al*, 1963).

During the inhibition process, thiocyanate was oxidised by lactoperoxidase to hypothiocyanite ($OSCN^-$) in the presence of sufficient hydrogen peroxide produced by the streptococci (Reiter, Pickering and Oram, 1964).

$$SCN^- + H_2O_2 \xrightarrow{\text{lactoperoxidase}} OSCN^- + H_2O$$

However, in no instance did the oxidation end product, produced in the absence of cells, ever show any inhibitory activity, which led to the suspicion that the active agent was an undefined intermediate oxidation product, and that this disappeared with increasing concentrations of hydrogen peroxide (Reiter *et al*, 1964). Two years later, in a study using streptococcal species of Lancefield groups A (*S. pyogenes*), K, L and N (*S. cremoris*), three intermediate oxidation products namely cyanate, "235 compound" (so called because it showed "increased extinction at 235mμ") and sulphite, were found (Oram and Reiter, 1966a,b). Glycolysis in the lactoperoxidase, thiocyanate sensitive *S. cremoris* 972 was inhibited by sulphur dicyanide but the same metabolic process in the lactoperoxidase, thiocyanate resistant *S. cremoris* 803 was not inhibited providing a source of energy was available. The latter strain was found to possess a "reversal factor" which, when extracted and partially purified, was able to reverse the inhibition of glycolysis of *S. cremoris* 972. The reversal factor also catalysed *in vitro* the oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) of the bacterial cell respiration reactions, in which all three intermediate oxidation products were found to be able to act as electron acceptors. The reversal factor was therefore termed the $NADH_2$ oxidising enzyme which, the authors speculated, might be active in other resistant species of bacteria.

The lactoperoxidase system can only function if all the constituents are available, and although lactoperoxidase is always present in milk, the levels of thiocyanate ions depend on the feeding regime (Reiter and Bramley, 1975). Staphylococcal and coliform organisms, which do not produce hydrogen peroxide, can only be inactivated by the lactoperoxidase system *in vitro* when an exogenous supply of hydrogen peroxide is available.

An explanation for the lack of viability of "streptococci of the scarlet fever type" in raw milk (Jones, 1928) compared with the growth, after a lag phase, of "the mastitis streptococcus" (Jones and Simms, 1929) followed studies on the effect of lactoperoxidase and thiocyanate on the growth of *S. pyogenes* and *S. agalactiae* in a chemically defined culture medium (Mickelson, 1966). Following a lag period of six hours *S. agalactiae* was able to obtain energy from glucose by utilising an alternative oxidative pathway, which was not lactoperoxidase, thiocyanate, hydrogen peroxide sensitive. Conversely, *S. pyogenes* could only obtain its energy by a fermentation pathway and was not able to use an oxidative pathway of glucose metabolism even in the presence of oxygen.

1.5.8 THE DEFENCE ABILITY OF DRY AND LACTATING QUARTERS AGAINST BACTERIAL INFECTION

Ten colony forming units of *Staph. aureus* suspended in 0.25ml sterile 0.85% saline, infused into the teat cisterns of all four quarters of five cows nearing the end of their first, second or third lactation, were sufficient to cause staphylococcal mastitis infection in all ten quarters (Reiter, Sharpe and Higgs, 1970). The pathogens were infused into the teat cisterns to ensure that infection rates were not influenced by the capability of the organisms to pass through the teat duct unaided. Six hours before the infusion of staphylococci, two of the quarters of each cow had been infused with 50ml saline which,

in the intervening six hours, caused those quarters' milk leucocyte counts to increase from less than 10,000 cells per ml, of which six per cent were neutrophils, to up to ten million cells per ml (85% neutrophils). The subsequent infusion of the ten colony forming units of *Staph. aureus* into the saline treated quarters failed to cause any intramammary infection, whereas all ten quarters not previously infused with saline became infected with staphylococcal mastitis within two days. In the same study, each of six cows had two quarters (one pair) dried off two weeks earlier than the other two quarters. Immediately after the second pairs of quarters had been dried off, 20 cfu of *Staph. aureus* were infused into the teat cistern of one quarter of all 12 pairs and 2,000 cfu of *Staph. aureus* were similarly infused into the remaining 12 quarters. Only one of the quarters dried off two weeks before being infused with 20 cfu of *Staph. aureus*, and one of the quarters dried off immediately before being infused with 20 cfu of *Staph. aureus* became infected, whereas all six quarters infused with 2,000 cfu of *Staph. aureus* immediately after being dried off became infected, as did four of the six quarters dried off two weeks before being infused with 2,000 cfu of *Staph. aureus*. The same experiment conducted on another six cows, but this time using *S. uberis* as the infectious agent, led to none of the 12 quarters that had been dried off immediately prior to the infusions of *S. uberis* (both 20 cfu and 2,000 cfu) becoming infected, whereas one of the six quarters dried off two weeks before infusion with 20 cfu of *S. uberis* and four out of six quarters dried off two weeks before infusion with 2,000 cfu of *S. uberis* became infected. Whilst there was sufficient oxygen in milk to fuel the destruction of *S. uberis* by the oxygen dependent lactoperoxidase, thiocyanate, hydrogen peroxide system during lactation, the authors thought that the increased ability of *S. uberis* to cause infection two weeks into the dry period resulted from the dry udder secretion which, although containing thiocyanate and peroxidase, did not contain sufficient oxygen to fuel the system and was, therefore, unable to destroy the invading *S. uberis.*

The growth of *S. agalactiae* in milk *in vitro* was stimulated by the addition of bovine mammary secretions collected at the start of the dry period (Brown and Mickelson, 1979); the addition of secretions collected as the dry period continued caused a progressive increase in the stimulation of *S. agalactiae* growth. This paralleled a corresponding rise in the concentrations of free cystine in the secretions confirming earlier work (Brown, 1974), which had shown that some sulphur containing compounds stimulated the growth of *S. agalactiae* in milk *in vitro*, the amino acid cystine being the most powerful agent used, with cysteine being less active; cystine and other sulphydryl residues could act as extra sites for the hypothiocyanite radical to react with (Aune and Thomas, 1978), thus reducing the concentration of hypothiocyanite available for attacking the bacteria (Marshall, Cole and Bramley, 1986).

Quarter mammary secretions collected 14 days before the end of lactation, seven days after drying off, and between three and 18 days post calving inhibited growth *in vitro* of *S. uberis*, but secretions collected 21 days after drying off showed no such inhibitory activity (Marshall *et al*, 1986). This reflected the susceptibility to *S. uberis* infection of the same quarters when infused with 250 colony forming units of the pathogen on each of the same four occasions; 43.8% of the quarters challenged 14 days before the end of lactation became infected, as did 25.0% challenged seven days after drying off, and 37.5% challenged in the period three to 18 days post calving, whereas 81.3% of quarters challenged 21 days after drying off became infected. Although the lactoperoxidase and thiocyanate concentrations were sufficient for antibacterial activity at 21 days post drying off, the authors concluded that the amount of hydrogen peroxide produced by *S. uberis* was insufficient, and that a source of hydrogen peroxide, such as from oxidase enzymes or the metabolic processes of viable somatic cells, was required for the lactoperoxidase thiocyanate system to have an antibacterial effect.

1.5.9 HUMORAL IMMUNITY

The immunoglobulins (Ig) found in ruminant mammary secretions are derived either from the blood serum (IgG), or are made locally by cells of the lymphocyte plasma cell series situated near the glandular epithelium (IgA and IgM) (Lascelles, 1979). With the exception of the time around parturition, the concentrations of the immunoglobulins in bovine serum were far greater than those in milk whey; the concentrations (in mg per 100ml) in serum, colostral whey and milk whey were measured as 1,400, 4,000 to 9,000, and 40 respectively for IgG_1, 1,300, 250 and six for IgG_2, 39, 470 and 11 for IgA, and 380, 540 and nine for IgM. The author concluded that "in this restricted immunological sense, the milk in the mammary gland is isolated from the rest of the body". The ratio of IgG_1 to IgG_2 in bovine colostrum far exceeded the comparable ratio in serum, and this ratio continued into lactation (Lascelles, 1979). However in the ewe, during the first few hours of an acute inflammatory insult such as the infusion of endotoxin or staphylococcal haemolysin into the udder (MacKenzie and Lascelles, 1968), the selective transfer of "fast IgG" (IgG_1) became inhibited to be replaced by a marked increase in the concentration of "slow IgG" (IgG_2) and serum proteins.

Although the concentrations of IgG_1 and IgG_2 in bovine blood have been found to be approximately equal, the major immunoglobulin in bovine colostrum and milk is IgG_1 and this is selectively transferred across the glandular epithelium into the mammary secretion in preference to IgG_2 (Brandon, Watson and Lascelles, 1971).

Immunoglobulin A in particular, and IgM, are synthesised locally in the mammary gland parenchyma (Lascelles, 1979). In comparison to the IgA synthesis in the female human, rabbit and guinea pig, IgA synthesis in the ruminant mammary gland is poorly developed and the immunoglobulin might only be used to cover bacterial adhesion sites and induce agglutination of bacteria, the latter being removed at the next milking.

Bactericidal activity of specific antibodies is mediated by complement and is active only against Gram negative organisms such as *E. coli* (Reiter and Bramley, 1975). Gram positive bacteria are not readily killed by the complement mediated bactericidal activity of specific antibodies; for these organisms antibody or complement together with neutrophils are required (Anderson, 1978).

1.5.10 PHAGOCYTOSIS BY POLYMORPHONUCLEAR LEUCOCYTES (NEUTROPHILS)

The predominant phagocytosing cell in the ruminant lactating mammary gland is the neutrophil (Jain, Schalm and Lasmanis, 1971), whilst in the non-lactating gland the large majority of cells are macrophages (Lee and Outteridge, 1976). Anderson (1978) reported that the life span of the neutrophil in the mammary gland was not known but was probably not more than a few hours, the cells with their ingested material degenerating and being removed by macrophages or expellation in the milk.

Bacteria are ingested into the neutrophil by invagination of the latter's cell membrane at the point of contact, which forms a phagosome. The bacteria are killed by intracellular granules (lysosomes) fusing their membranes with those of the phagosome and discharging their contents into the phagolysosome (Anderson, 1978). Primary granules discharge myeloperoxidase which, with hydrogen peroxide (provided by the respiratory burst associated with ingestion) and chloride ions, complete the bactericidal myeloperoxidase, hydrogen peroxide, halide system; they also discharge lysozyme, elastase and cationic proteins, which are oxygen independent bactericidal reagents. Secondary granules discharge lactoferrin and lysozyme into the phagolysosomes. Once a neutrophil has degranulated no new granules are formed in the same cell.

However, the efficiency of the ingestion and killing of bacteria by the neutrophil in the mammary gland is not infallible as evidenced by the chronic nature of many mastitis infections (Paape, Wergin, Guildry and Pearson, 1979). The phagocytic activity of neutrophils isolated from milk was reported to be lower than from neutrophils isolated from blood (Wisniowski, Ronaniukowa and Grajewski, 1965), and microscope examination showed that 44% of blood neutrophils had de-activated (adhered to or phagocytosed) an average of 1.6 *Staph. aureus* whereas only 30% of milk neutrophils had de-activated an average of 1.08 *Staph. aureus* (Kent and Newbould, 1969).

The reasons given for the poor performance of neutrophils in milk have included low energy levels, deficiency of opsonins (the immunoglobulins that bind to foreign bodies in order to make them more readily phagocytosed by neutrophils), and the phagocytosis of milk components (Paape *et al*, 1979).

The glycogen levels of blood neutrophils and milk neutrophils of five cows all at different stages of their reproductive and lactation cycles were measured by Naidu and Newbould (1973). Milk samples for the neutrophil analysis were collected on the four days following each of four weekly infusions of sterile sodium chloride solution containing *E. coli* lipopolysaccharide. Blood samples were "routinely collected" from the same five cows, however there was no evidence that the blood samples were collected during the four weeks of the milk sampling. It was found that 97 to 98% of the milk leucocytes were neutrophils and that the overall milk leucocyte glycogen level was 3.81mg per 10^9 cells. This was significantly lower than the blood leucocyte glycogen level of 6.11mg per 10^9 cells.

Wisniowski *et al* (1965) reported that a deficiency in opsonins could contribute to reduced phagocytosis by leucocytes. The ability of leucocytes, obtained from bovine mammary gland secretions following irritation of the glands by infusion of sterile

distilled water, to ingest staphylococci was increased by up to 18% when one per cent of immune serum was added to the whey containing the bacteria and leucocytes (Newbould, 1973); little improvement on this performance was seen when more serum was added. The addition of 20mg% glucose to the whey caused an increase in the phagocytosis of up to 20%, and adding 40 and 60mg% glucose gave further small increases. Little advantage was obtained by combining serum and glucose at any of the levels studied.

The ability of whey, collected from 12 cows in their first, second or third lactation, to support phagocytosis of live *Staph. aureus* after the cows had been immunised with multiple intramammary infusions of heat killed *Staph. aureus* was compared with the phagocytosing ability of whey collected from the same cows before immunisation (Guidry, Paape and Pearson, 1977). Neutrophil phagocytosis of the staphylococci increased by 62% following immunisation. Absorption of the immune whey with homologous *Staph. aureus* removed the opsonising effects of immunisation.

Neutrophils isolated from the mammary secretions of ewes immunised with either subcutaneous injections of live *Staph. aureus* or intramuscular injections of a killed *Staph. aureus* oil adjuvant vaccine were used in phagocytosis assays against *Pseudomonas* species and *Staph. aureus* (Watson, 1976). The neutrophils from both groups of ewes showed no difference in their ability to phagocytose the *Pseudomonas* species bacteria but the neutrophils from the ewes that had received the live vaccine had significantly greater phagocytic activity against *Staph. aureus* than neutrophils from the ewes that had received the killed vaccine. Removal of the cytophilic antibodies, which were found to be IgG_2, abrogated the superiority of the neutrophils from the live vaccinated ewes, and it was restored by replacement of the antibodies. When neutrophils from non-immunised ewes were coated with purified IgG_2 from the sera of the live vaccinated ewes they exhibited enhanced phagocytosis of staphylococci

compared with neutrophils from non-immunised ewes coated with IgG_2 from the sera of ewes given the killed vaccine.

In a similar study on bovine milk neutrophils, removal of the cytophilic antibodies caused the neutrophils to clump together (Paape, Pearson and Schultze, 1978). It was thought that this was due to the phagocytosed casein and fat globules in the milk neutrophils causing a cascade of intracellular and extracellular events, amongst which was an increased tendency to clump.

The fat and casein in milk have also been shown to have a detrimental effect on the phagocytic performance of neutrophils (Paape, Guidry, Kirk and Bolt, 1975). Neutrophils, recovered from bovine blood, phagocytosed 74% of killed *Staph. aureus* when both were incubated together in skim milk and 72% when the skim milk was replaced by whey, but only 44% of the *Staph. aureus* were phagocytosed when the incubating medium was whole milk. Milk neutrophils, obtained from milking a quarter stimulated 12 hours previously with an infusion of sterile phosphate buffered saline containing 0.1% oyster glycogen showed a similar performance, 72%, 74% and 44% of *Staph. aureus* being phagocytosed respectively. Phagocytosis decreased when cream was added to the skim milk. A greater proportion of viable staphylococci were able to be recovered from neutrophils after both had been incubated together in whole milk compared to being incubated in skim milk (Paape and Guidry, 1977); this was attributed to phagocytosed milk fat globules causing degranulation of the primary and secondary lysosomes. Ingested casein was also found to have a detrimental effect on the ability of both milk and blood neutrophils to phagocytose *Staph. aureus* (Russell and Reiter, 1975).

Both electron microscopy studies and evidence of increased peroxidase content of phagolysosomes containing fat and casein pointed to the fact that lysosomes discharged

their contents into the fat and casein phagocyte vacuoles thus diverting, as well as neutralising, the bactericidal agents from vacuoles containing bacteria (Russell, Brooker and Reiter, 1977; Paape and Wergin, 1977a,b).

In a comparison of the surface structure of the neutrophils isolated from blood and milk, 98% of blood leucocytes, of which 53% were neutrophils, showed well developed pseudopod formation over more than 50% of the cell surface (Paape *et al*, 1979) whilst only 37% of milk neutrophils showed the same feature over the same proportion of cell surface. The authors suggested that the phagocytosis of fat globules contributed to the loss of pseudopodia from milk leucocytes, which gave the smooth surface structure characteristic of the cells.

1.6 MASTITIS CONTROL

1.6.1 THE EARLY INFLUENCE OF MILKING MACHINES ON UDDER INFECTION

The authors of one of the earliest reports on an attempt to control an outbreak of acute contagious mastitis, which had appeared after the introduction of machine milking, stated that 75% of a 40 cow herd had contracted mastitis in one or more quarters (Gilruth and MacDonald, 1911); *Bacillus lactis aerogenes* (*B. lactis aerogenes*) was isolated from secretions collected from two of the infected udders. The milking machine teat cups, which were only washed with cold water after each milking and placed in lime-water overnight once a week, were "very dirty, one containing a small dried splash of manure". The lack of cleanliness was judged to be responsible for the spread of the infection. No treatment or control measures were recorded except that the use of the milking machine was "temporarily discarded".

1.6.2 MILKING PROCEDURES AND SEGREGATION OF INFECTED COWS

The cases of bovine mastitis in 280 cows from four machine milked herds were recorded for a period of two and a half years following the introduction of hand milking for all cows that were clinically infected with mastitis after the rest of the herd had been milked, and the milkers washing their hands between handling each infected udder (Hardenbergh and Schlotthauer, 1927). In the last six months of 1924 there were 25 cases of clinical mastitis (no figures were recorded for the twelve months of that year). The number of clinical cases for the whole of 1925 was 34, and this dropped to only nine cases in the following year. Streptococci were isolated from milk samples collected from 40 of the cases, staphylococci from seven cases, *B. coli* from six cases, a mixture of staphylococci and streptococci from six cases and, in the majority of the other cases, the organisms either did not grow on culture or were not sufficiently identified. In addition to the milking hygiene measures, "indiscretions of diet are avoided as much as possible, the cows are milked at regular intervals, and clean stalls and abundant litter are provided. All unnecessary trauma to the udder and teats, no matter how slight, is avoided".

The spread of mastitis caused by infection with group I streptococci (Edwards, 1932) (*S. agalactiae*) in a hand-milked herd of 53 cows, in which the milkers washed their hands between milking each cow, was controlled by infected cows being milked after the rest of the herd (Minett *et al*, 1933). Later, infected cows were removed from the dairy herd altogether to form a separate suckler herd, and both herds were moved on to fresh ground; no infected cow ever rejoined the 'uninfected' dairy herd. Within two years *S. agalactiae* could not be isolated from milk samples routinely collected for bacterial examination, and this continued for a further three years. Although the dairy herd was reported to be free from *S. agalactiae* infection, group II streptococci (Edwards, 1932) (*S. dysgalactiae*) and group III streptococci (Edwards, 1932) (*S. uberis*) were

still isolated on many occasions. The enriching medium, in which milk samples were incubated prior to being subcultured on blood agar, contained crystal violet (concentration 1:200,000) in order to "inhibit the growth of many of the organisms normally present in milk, in particular staphylococci, without interfering with the development of streptococci"; any infections caused by staphylococci could not, therefore, be recorded.

1.6.3 AGE AND INFECTION

Monitoring a herd of 45 to 50 cows for three years showed that the probability of a cow contracting mastitis in the dry period increased with the age of the cow (Neave, Dodd and Henriques, 1950). However in a herd of 94 heifers, the incidence of clinical and subclinical mastitis at first calving, and during the subsequent lactation and dry period, was directly proportional to the speed at which the heifers milked out, and hence a direct relationship was established between the ability of bacteria to enter the udder and the size of the teat canal (Dodd and Neave, 1951). It was suggested that the susceptibility to mastitis of older cows might be due to the fact that cows with narrower teat canals, and thus greater resistance to mastitis, were being culled at an early age purely due to their slowness of milking thus leaving the faster milking, but more mastitis susceptible, cows in the herd. Evidence that faster milking cows were more likely to develop mastitis from the trauma of having milking machines left on longer than necessary was discounted as, by the same reasoning, the smaller front quarters, holding less milk than the rear quarters, would have a higher incidence of mastitis that was not the case. Bacterial analysis of milk samples collected from six heifers which had mastitis at their first calving, showed that four of the infections were caused by *S. uberis*, one by *S. dysgalactiae* and one by a coliform bacterium.

1.6.4 INFECTION IN DOWN-CALVING HEIFERS

Heifers were presumed to be free from mastitis until after their first calving and "the incidence of the disease rises with age when animals are exposed to infection" (Minett *et al*, 1933). However, the same authors also noted that group II streptococci (Edwards, 1932) (*S. dysgalactiae*) had been isolated "in very large numbers and in apparently pure culture" from the watery udder secretion collected from a heifer before her first parturition.

Miller (1936) questioned whether heifers' mammary glands could be infected prior to first calving and cited the hypothesis, current at the time, that a calf's prepubescent udder could be infected through having its teats sucked by penmates that had recently drunk milk infected with streptococci, the infection remaining latent until the udder began to function. To support this theory, streptococci were isolated from the colostrum of one or more quarter samples from 27 of 100 first calving heifers, the colostrum being collected immediately after parturition and before the calf had sucked. Eight of 20 heifers, from which a second sample was taken "within a short time" of the first and "before the animal was placed in the milking herd", remained infected. Thirty-eight strains of streptococci were isolated; apart from six strains of *S. agalactiae*, three of which were isolated from three heifers at the first sampling and three from three different heifers at the second sampling, 25 of the remaining 32 strains were mannite positive, and the majority of these hydrolysed aesculin (17 strains) and sodium hippurate (21 strains). No specific identification of these strains was made but the author noted that their properties were similar to those of group III (Edwards, 1932) and group Ba (Plastridge *et al*, 1932).

Mastitis in precalving heifers caused by cross sucking by calves was further implicated from a study involving three herds of cows in which the prevalence of *S. agalactiae*

mastitis at the beginning of the study ranged from 25.6% to 54.0% (Schalm, 1942). Differential media tests on milk samples, collected from 248 heifers during the first week of lactation, showed that eight heifers (3.2%) were infected with *S. agalactiae*, four (1.6%) with *S. uberis*, two (0.8%) with *S. dysgalactiae*, and 16 (6.4%) with an "atypical" streptococcus, the latter disappearing from the udder within three weeks of parturition. Six of the eight heifers infected with *S. agalactiae* were in the herd which had the 54% prevalence of *S. agalactiae*, and two of them remained infected throughout their first lactation and into their second lactation. Although the calves in all three herds were removed from their dams within 24 hours of birth, they were fed unpasteurized milk from the herd until they were four to five months old. Some calves developed the habit of sucking the teats of their penmates, most noticeably immediately after feeding. The author also monitored calves in another herd with a prevalence of *S. agalactiae* mastitis of 54.5%, where only pasteurized milk was fed, and any calf showing a tendency to suck its penmates was muzzled. None of the 36 heifers, raised by this system, that calved during the period of the study showed *S. agalactiae* infection in the first month of lactation.

In the same study (Schalm, 1942), calves' oral mucosae were swabbed immediately after being fed infected milk (containing between 65,000 and 585,000 *S. agalactiae* per ml) and at 15 minute intervals thereafter for two hours. No *S. agalactiae* could be grown from swabs collected later than 45 minutes after feeding, the charged swabs having first been incubated in ten millilitres sterile broth at 37°C for one hour, 0.01ml of this broth then being mixed with ten millilitres sterile saline of which 0.5 ml was plated on to blood agar. *Streptococcus agalactiae* was also recovered from the teats of one of four calves which had been sucked by penmates that had just consumed infected milk. The author noted that other workers (Klein and Kleckner, 1941) had found that, by streaking the charged swabs directly on to blood agar, *S. agalactiae* was regularly

isolated from the oral cavity of calves four hours, and sometimes up to 14 hours, after being fed with infected milk.

Less conclusive results were produced from the study of two groups of 14 heifer calves fed for several months on "liberal" amounts of unpasteurized milk from eight cows with mastitis, *S. agalactiae* having been isolated from 11 quarters and haemolytic staphylococci from nine quarters (Johnson, 1947); the secretions from 14 of the quarters contained flakes, clots or pus, or had a watery consistency. From the group of calves that had been penned individually, twelve heifers eventually calved of which six produced abnormal milk for one to three milkings; whilst no *S. agalactiae* was recovered from their milk, one quarter was infected with *S. uberis*. Twelve quarters from another five heifers in the same group produced milk with "large numbers" of haemolytic staphylococci and a leucocyte count of over one million cells per ml. From the other group of calves that had been fed a similar diet of infected milk but had been allowed to suck each others teats, only six heifers calved during the period of the study. The only pathogen recovered was *S. agalactiae* from one heifer at the first examination after calving, all other samples being negative.

1.6.5 SUBCLINICAL INFECTION

From the late 1940's onward, the importance of subclinical mastitis was increasingly appreciated: Subclinical streptococcal and haemolytic staphylococcal infections were found to be able to persist in the bovine udder for several months, and cause a gradual reduction in milk yield and quality (Crossman *et al*, 1950).

Results from two "random samples" carried out in two counties in England in 1948 and 1960 showed that the prevalence of *S. agalactiae* had only dropped two per cent from 14% in 1948 when penicillin and other antibacterial agents were neither readily available

or widely used, to 12% in 1960 (Wilson, 1963). As *S. agalactiae* responded readily to antibacterial therapy, the failure to eradicate the infection was blamed on farmers' lack of awareness of the fact that mastitis was a herd problem which required whole herd treatment and that *S. agalactiae* could not be eradicated merely by treating those cows showing clinical signs.

Evidence from field trials using over 2,900 cows showed that two-thirds of mastitis infections remained subclinical, hence unnoticed and untreated, and would remain a source of infection for other cows in the herd (Neave, Dodd and Kingwill, 1966). Data from the Ministry of Agriculture, Fisheries and Food Mastitis Surveillance Scheme which monitored 400 dairy herds in England and Wales during the first five months of 1980 showed that, on a monthly basis, 16.6 to 21.4% of all cases of clinical mastitis were due to *S. uberis* (Wilson, 1981); according to Bramley (1984), these cases came from more than 70% of herds investigated and, in addition, five per cent of cows were subclinically infected. Since the early 1970's in the United Kingdom, measurement of the milk somatic cell count of a herd (bulk tank milk somatic cell count) has been used as a measure of the level of subclinical mastitis in a herd (Booth, 1991). With the introduction of bulk tank milk somatic cell count testing by all the milk boards with penalties being exacted when the cell count exceeds a certain level which, over the foreseeable future, will continue to be lowered, the economic implications of subclinical mastitis have become a growing problem to the dairy farmer (Booth, 1993).

1.6.6 DRY PERIOD INFECTIONS

Cows with infected quarters at the end of lactation were more likely to experience new infections during the dry period than cows free of infection at the end of lactation (Neave *et al*, 1950), and dry period infections were more persistent in cows already infected than in uninfected cows. In one herd, 33 (50%) of 66 dry period infections in cows not seen

to be infected at the end of the previous lactation, were caused by viridans streptococci, the majority of the infections being detected within 21 days of the cow being dried off. The spontaneous disappearance of many of these infections in the late dry period or early lactation was thought to be due to environmental changes within the udder.

The occurrence of dry period infections was directly related to the milk yield being given when the udder was dried off (Oliver, Dodd and Neave, 1956a). In a herd of 113 cows, 30 (18%) of 163 quarters from 20 (42%) of 48 cows giving less than seven pounds (three kilograms) milk at the end of lactation became infected during the dry period, whereas 23 (43%) of 53 quarters from ten (71%) of 14 cows giving over 21lb (9.5kg) milk were found to have new infections in the dry period. "Green" streptococci, which included *S. dysgalactiae*, *S. uberis* and Lancefield group D streptococci, were isolated from 58 of 108 new dry period infections; the number of infections due to each species was not recorded.

When an average of 580 cfu of *Staph. aureus* were infused through the ducts of 12 teats of five cows that were milked twice daily, just one quarter became infected (Newbould and Neave, 1965). Five quarters became infected following the infusion of an average of 520 cfu *Staph. aureus* into 19 quarters of five cows that were then not milked for the 24 hours following infusion.

Quarters that had been dried off for up to 21 days were far more susceptible to mastitis than quarters that were still being milked (Neave, Oliver, Dodd and Higgs, 1968). Two quarters of each udder of six cows nearing the end of their lactation were dried off three weeks before the rest of the quarters (control quarters). During those three weeks all the quarters were dipped twice daily in a suspension of litmus milk containing *Staph. aureus*, *S. dysgalactiae* and *S. uberis*. None of the milking quarters developed mastitis, but seven of the 12 dry quarters became infected, four with *Staph. aureus* and

three with a mixed infection of *Staph. aureus* and *S. uberis*. From evidence provided by earlier work (Dodd and Neave, 1951; Oliver *et al*, 1956a; Newbould and Neave, 1965), it was concluded that the high internal pressure that built up in the udder for the few days following drying off, with milk seeping through the streak canal, would allow bacteria to track through the streak canal and enter the udder, a process that, during lactation, would be arrested by the flushing action of twice daily milking.

1.6.7 TEAT DISINFECTION AT THE END OF LACTATION

Significant reductions in new infections of *Staph. aureus* during the dry period were achieved when, after the last milking of the lactation, the teats were washed with a solution of sodium hypochlorite containing 800 parts per million (ppm) available chlorine, dried and then dipped in a five per cent solution of tincture of iodine for 20 seconds, this being repeated 24 hours later (Oliver, Dodd and Neave, 1956b). However, this routine had no beneficial effect on new dry period infections caused by *S. uberis.*

When 37 quarters, one each from 37 cows, were dipped in a mixed culture of *Staph. aureus* and *S. uberis*, and then dipped in a two per cent solution of chlorhexidine (undiluted "Hibitane Udder Wash"; ICI) for 20 seconds following the last milking of the lactation, only one quarter contracted *Staph. aureus* infection (Oliver, Neave and Sharpe, 1962), however subsequent dry period infections by *S. uberis* were not so well controlled, six quarters being infected including the quarter infected by *Staph. aureus*, although it could not be established whether the *S. uberis* isolated from the infected quarters was the same strain as that used to deliberately contaminate the teats.

After the last milking of the lactation, 78 teats (from 28 heifers in the text, 20 heifers in the table) were dipped in "naturally infected milk" or in a solution containing varying

dilutions of *Staph. aureus*, *S. agalactiae* and *S. dysgalactiae* (Neave and Oliver, 1962). For two to three weeks before being dried off, the skin of the teats and udders had been washed with sodium hypochlorite (800 ppm available chlorine) both before and after milking and the teat cup clusters had been disinfected by heat or chemical means between milking each animal. Those quarters that had received a heavy contamination of *Staph. aureus* suffered far more dry period infections than lightly contaminated quarters. *Streptococcus uberis* was not one of the pathogens used to artificially contaminate the teats and, although it was not recovered from either teats or teat orifices at the end of lactation, it was present in large numbers in six teat orifices 21 days later and all were associated with infected quarters, three as a pure *S. uberis* infection and three as a mixed infection with *Staph. aureus*. It was assumed that the *S. uberis* had come from the environment of the dry cows.

1.6.8 ROUTINE MILKING HYGIENE MEASURES

Work carried out at the National Institute for Research in Dairying at Shinfield, England, showed that hand and udder washing with disinfectant solution containing 100 ppm chlorhexidine digluconate ("ICI udder wash") or 100 ppm available iodine ("Iosan CCT"; Ciba-Geigy Agrochemicals, now Ciba Animal Health, Whittlesford, Cambridge, CB2 4QT, England) using individual udder cloths or disposable paper towels, and post milking teat dipping with a disinfectant (5,000 ppm chlorhexidine digluconate, or 5,000 ppm available iodine) reduced the mastitis incidence rate by more than 50% (Neave *et al*, 1966). Teat cup pasteurization (at 85°C) between each cow was evaluated but found not to cause a significant decrease in the infection rate to warrant its considerable additional expense. Other recommendations were the wearing of gloves by the milker, examination of the foremilk with a strip cup, treatment of clinical mastitis cases with antibacterial therapy, dry cow antibacterial therapy for every quarter of every cow at the end of lactation, and culling cows prone to recurrent mastitis.

From these principles a set of routine hygiene measures were proposed for the control of mastitis (Kingwill, Neave, Dodd, Griffin, Westgarth and Wilson, 1970; Wilson and Kingwill, 1975). These included pre-milking udder and teat washing with disinfectant solution or clean running water, post-milking teat dipping, appropriate antibiotic treatment for clinical mastitis cases, records to be kept of each case of mastitis so that cows with recurrent infection could be culled, routine dry cow therapy for all cows, and regular milking machine maintenance. Compared with treatment given during lactation, the advantages of dry cow therapy for all quarters at the end of lactation included higher rates of elimination of both clinical and subclinical infection without the economic loss from unsaleable milk, and prevention of new infections starting during the dry period. Using these hygiene measures in 30 herds ranging in size from 40 to 90 cows and encompassing various systems of housing and management, the incidence of subclinical and clinical mastitis caused by staphylococcal and streptococcal infection was markedly reduced within one year (Kingwill *et al*, 1970). However, whilst subclinical staphylococcal infection continued to fall in the second year, there was little further decrease in subclinical streptococcal infections. Of the streptococcal species isolated, only *S. agalactiae* was identified by name, and this was shown to have been controlled in the first year. There was no explanation for the lack of continuing control of subclinical infection caused by other unnamed streptococcal species in the second year.

1.6.9 ANTIBACTERIAL THERAPY

In the early part of the twentieth century, before the introduction of modern antibacterial and antibiotic agents, many different substances were used in an attempt to find an effective bactericidal agent against mastitis infection. Conventional remedies such as boric acid, oxygen gas, and equal parts of alcohol and glycerine injected into the udder were unsuccessful (Frost, 1917). Frost (1917) was of the opinion that, as respiratory and urino-genital tract infections were treated with antiseptics given by mouth and

eliminated through the infected organ, it should have been possible to treat infections of the udder in the same way and, in cases of mild mastitis, he reported obtaining "good results" with oral administration of methylene blue with and without the addition of turpentine. Realising that "something stronger was needed", administration of formalin by mouth gave "gratifying" results and, after some preliminary tests, he reported that giving up to one ounce of formalin daily produced a cure in cases of bovine mastitis caused by streptococci, micrococci and *B. coli.*

According to Johnson (1941) the first therapeutic agents to be infused into udders with mastitis were "simple disinfectants such as therapogen, creolin, methylene blue, and others (which) offered little help of success": Bugge (1924) was reported to have used the less tissue irritating and more bactericidal acridine derivative rivanol, and after comparing other acridine derivatives Schnorf (1925) advocated uberasan: Uberasan was a derivative of rivanol (Schalm, 1940).

Stableforth and Scorgie (1938) reported the results of several workers who had infused various substances into quarters affected by mastitis: Hucker and Lee (1932) found crystal violet, brilliant green and acriflavine to be of no value, but obtained promising results with azamine. After comparing various preparations including sulphurous acid, Seelemann and Siemonsen (1933) found the acridine derivatives entozon and rivanol "definitely superior to the others" due to their high bacterial reaction on streptococci with comparatively low tissue reaction. In a comparison of the use of uberasan, rivanol, entozon and tryptoflavine over a period of five years, Steck (1934) reported that "at concentrations of equal irritant action on the udder, tryptoflavine had the highest bactericidal action in normal or slightly altered secretions whilst entozon excelled in pus-like secretions".

Attempts at other forms of treatment were also reported: As ultraviolet light was "known to have a curative effect on rickets.....and on certain skin diseases" Gildow, Hanson and Cherrington (1933) used a "Cooper-Hewitt-Uviarc poultry treater" to shine ultraviolet light on affected udders for 15 minutes twice daily for five days before milking. After a break of three days the procedure was repeated although this time it was carried out after milking. Although the treatment was reported to produce a reduction in the clinical signs, the bacteria and leucocyte counts of the milk remained high.

With time, it was recognised that simple antiseptics such as phenols, cresols, formalin, and salts of the heavy metals were too irritant to be used (Stableforth and Scorgie, 1938), although "Schnorf, Diernhofer, Seelemann, Steck and other investigators were reporting varying degrees of success in the use of rivanol, uberasan, entozon and tryptoflavine" (Johnson, 1941): Tryptoflavine, which was manufactured in Germany, was "exactly similar" to acriflavine which was made in Great Britain (Stableforth and Scorgie, 1938).

To compare the effect of entozon (concentration 1:1,250) and acriflavine (concentration 1:10,000) on quarters infected with clinical or latent (subclinical or chronic) *S. agalactiae* mastitis, an infusion of 500 to 1,500ml of either substance was pumped into infected milking quarters and stripped out five minutes later; the same procedure was repeated after 24 hours (Stableforth and Scorgie, 1938). Acriflavine was initially administered at a concentration of 1:1,000 but it caused termination of milk secretion and atrophy of the quarter. The infusion was left in infected dry quarters for 24 hours and repeated after one week. The bacterial content of the milk was subsequently examined after it had been incubated on blood agar and Edwards agar. Entozon was reported to have "cured" 13 of 37 lactating quarters affected with clinical mastitis, and 43 of 60 quarters with latent mastitis; the figures for acriflavine were nine of 14, and

26 of 29 quarters respectively. Similar results using entozon (concentration 1:1250) were reported by Schalm (1940), and the majority of cases caused by *S. agalactiae*, as well as some cases caused by *S. dysgalactiae*, *S. uberis* and *Staph. aureus*, responded to treatment with acriflavine (concentration 1:8,000 or 1:4,000) (Johnson, 1941).

1.6.10 SULPHONAMIDE THERAPY

The acceptability of sulphonamide therapy as a veterinary antibacterial agent grew in the latter half of the 1930's with the use of the original compound sulphanilamide (Scheidy, 1948); other related compounds were also used. Many workers showed that the oral administration of sulphanilamide was ineffective in eliminating streptococci permanently from the infected udder "even in cases where the doses were large enough to produce toxic effects" (Kakavas *et al*, 1942). However, by infusing sulphanilamide, which had been homogenised in "light, liquid petrolatum" directly into infected quarters via the teat canal, Kakavas *et al* (1942) "cured" 251 of 265 quarters from 92 of 103 cows, 100 of the cows being infected with *S. agalactiae* and three with *S. uberis*. Results from preliminary trials on cases of staphylococcal mastitis were reported as being "promising". In the United Kingdom, treatment of streptococcal mastitis with sulphanilamide produced clinical recovery in a high percentage of infections but a bacteriological "cure" occurred in relatively few cases (Barrett, 1945), leading to the possibility of a relapse during the same or future lactations with the danger of spread of the infection to other cows.

Sulphanilamide at a concentration of 20mg per cent in tryptose broth had no bactericidal effect on Lancefield group B streptococci (*S. agalactiae*) at 37°C, but destroyed the pathogen at 40.5°C (Kakavas, 1945). At 37.5°C it was necessary to have a concentration of over 100mg per cent sulphanilamide to obtain a bactericidal action, and this concentration had to be maintained during the early bacterial growth phase.

Following work which had shown that para-amino benzoic acid (PABA) possessed antisulphanilamide activity (Woods and Fildes, 1940), one part by weight of PABA was found to nullify the germicidal effects of 25 parts by weight of sulphanilamide for one particular strain of *S. agalactiae* (Kalkavas, 1945). In a later experiment, the growth of *S. agalactiae* was not impeded by the presence of PABA and sulphanilamide in a ratio of 1:1,000 (Edwards, 1949), and "the drug only becomes effective when the multiplying organism is unable to synthesise PABA at a sufficient rate to antagonise the competitive action of sulphanilamide".

1.6.11 PENICILLIN

Following the discovery of penicillin (Fleming, 1929), and its purification, standardisation and clinical application (Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing and Sanders, 1940; Abraham, Chain, Fletcher, Gardner, Heatley, Jennings and Florey, 1941; and others), work was directed into its application as a treatment for bovine mastitis. In one of the earliest studies (Kakavas, 1944), 23 quarters infected with *S. agalactiae* were given between 600 and 1,200 Oxford units of "raw" penicillin in whey broth through the teat canal daily for three to eight days, and the bacterium was eliminated from 21 of the quarters, this assertion being based on "repeated testing of the milk taken at two and four weeks after the last dose had been administered". The "Oxford unit" was defined as "that amount of penicillin which when dissolved in 50ml of meat extract just inhibits completely the growth of the test strain (Oxford strain) of *Staph. aureus*" (Florey and Jennings, 1942). *Staphylococcus aureus* was similarly eradicated from 16 out of 27 infected quarters, using three or four daily doses of between 600 and 1,000 Oxford units per quarter although one quarter was given 2,300 Oxford units per dose (Kakavas, 1944). In the same study, ten of 15 infections with *S. agalactiae*, all six with *S. dysgalactiae*, all three with *S. uberis* and all four with streptococci of Lancefield group D were successfully treated with intramammary

infusions of sodium penicillin, however only four of seven cases of *Staph. aureus* mastitis responded to the same treatment; the dose of penicillin and the length of treatment varied with each case.

Four quarters infected with staphylococcal mastitis were treated with eight intramammary infusions of 22,800 Oxford units of penicillin at intervals of six hours, and three similarly affected quarters with four infusions at intervals of 12 hours (Klein, Crisman and Moor, 1945). Milk samples collected from the quarters were free of staphylococcal organisms for the first two days after the last infusion, but six quarters showed a return of infection by the sixth day after the last infusion. The one other quarter, which had received the eight six-hourly injections, remained free of the original haemolytic staphylococci for the full 13 days of the study.

The *in vitro* sensitivity to penicillin of 150 bacterial cultures isolated from udder secretions of cows with "varying degrees of mastitis" was investigated by streaking the cultures on blood agar containing various known concentrations of penicillin (Heishman, 1947). All 53 strains of *S. agalactiae* and all 22 strains of *S. uberis* were sensitive to a concentration of 1.125 units penicillin per ml, and three strains of *S. dysgalactiae* to 0.031 units per ml agar; it was not stated whether Oxford or international units were being used. Fifty of the 52 strains of *Staph. aureus* were inhibited by 0.25 units per ml, one strain grew slightly at 0.25 units per ml, and one strain grew well at two units per ml. Four strains of *Corynebacterium pyogenes* (now *Actinomyces pyogenes*) were inhibited by concentrations of between 0.015 and 0.062 units per ml. Ten rod-shaped organisms thought to be coliform bacteria failed to be inhibited at the highest concentration of two units per ml.

Sixteen strains of *S. agalactiae* were susceptible to 0.06 to 0.25 units penicillin per ml of Wrights broth or autoclaved fresh milk (Brownlee, 1945); it was not stated whether

Oxford or international units were being used. Fifty-six strains of *S. agalactiae* and four strains of haemolytic *Staph. aureus*, all from clinical cases of mastitis were sensitive to 0.04 Oxford units penicillin per ml of sterilised skim milk (with litmus milk added as an indicator of bacterial growth) (Watts and McLeod, 1946). Twenty of 25 strains of *S. agalactiae* were susceptible to 0.04 to 0.06 Oxford units penicillin per ml of sterilised milk with another four strains susceptible to 0.1 Oxford units per ml and the remaining strain to 0.2 Oxford units per ml (Edwards, 1949). The efficacy of ten Oxford units penicillin per ml of fresh milk was no greater than that of 0.06 Oxford units per ml and the rate of destruction of *S. agalactiae* did not increase with concentration of the penicillin.

All 122 isolates of *S. agalactiae*, all 89 isolates of *S. dysgalactiae*, 116 of 127 isolates of *Staph. pyogenes* and 96 of 98 isolates of *S. uberis* were inhibited by two Oxford units of crystalline sodium penicillin per ml of blood agar (Aynsley, 1953); the other 11 isolates of *Staph. pyogenes* were sensitive to 2.5 Oxford units penicillin per ml and the other two isolates of *S. uberis* were only inhibited at a concentration of five Oxford units per ml. Other *in vitro* studies showed that isolates of *S. uberis* were susceptible to between 0.031 and 0.063 Oxford units penicillin per ml of blood agar (Throop and Swanson, 1958), and 0.01 international units (iu) penicillin per ml blood agar (Pedersen, 1960).

"Blitz" treatment of 25 herds of cows with penicillin (no details of preparation or treatment regime were given) was reported to have "removed" *S. agalactiae* infection from the herds (Wilson, 1954); no details of the bacterial examination of samples was recorded. When the same herds were tested four years later (no details of the numbers of cows entering or leaving the herd in the intervening period were given), *S. agalactiae* was isolated from only 11 milk samples, compared with 338 cases originally. Over the same period, the prevalence of *S. dysgalactiae* infections had declined from 58 to 40

and *Staph. pyogenes* infections had risen from 81 to 86. However, 52 cases of *S. uberis* infection at the time of the "blitz" treatment time had risen to 117 cases four years later. Two other herds, both maintained at the Central Veterinary Laboratory, Weybridge, England, had also been given mass treatment with penicillin: In one herd, *S. dysgalactiae*, which had been the predominant udder pathogen, was not isolated for two years following treatment but then reappeared even though no new animals had been taken into the herd. In the second herd, the main pathogen isolated was *S. uberis*, and the "blitz" treatment only reduced the "infection rate" (not defined), and the disease quickly re-established itself when the treatment was discontinued. Nine years later, Wilson (1963) stated that *S. uberis* was "best treated with large doses of penicillin and preferably by the systemic route in the severe cases".

Streptococcus uberis, isolated from an udder which had remained infected despite repeated treatment (four successive monthly treatments of four daily infusions of 50,000 Oxford units of penicillin), was inoculated into three quarters of an uninfected udder (Throop and Swanson, 1958). Within 72 hours, the recipient cow had acute clinical mastitis in the three inoculated quarters; the uninoculated quarter remained free of infection. Twenty-four hours after a four day course of daily infusions of 50,000 Oxford units of penicillin, in distilled water, into each infected quarter there was a "marked improvement" and by 72 hours the "leucocyte content of the milk had approached normal and the milk yield was increasing", however no pre- or post-infection milk somatic cell counts were recorded. No further *S. uberis* was isolated from the recipient udder before slaughter four months later. The penicillin treatment was thought to have failed in the original cow because an "adequate amount" of the antibiotic was unable to reach all the foci in the established infection.

Bacterial analysis was carried out on all milk samples collected weekly from each lactating quarter, and from all four quarters of an udder in all cases of clinical mastitis,

during a complete lactation of the 24 cows which made up one of the herds at the CVL (Sweeney, 1964). All cases of clinical and, when found, subclinical *S. uberis* mastitis were treated with four alternate day infusions of 100,000 units of procaine penicillin into the teat canal. The cure rate was not stated but clinical *S. uberis* infections were reported to be "readily eliminated".

1.6.12 AQUEOUS VERSUS OIL BASED PREPARATIONS AND THE DEVELOPMENT OF DRY COW PREPARATIONS

When using intramammary infusions of acriflavine, one of the acrinidine disinfectants, as a treatment for mastitis, Johnson (1941) noted that "some of the troublesome cases (of mastitis) that would not have reacted favourably to treatment during the milking period, were freed from infection when treated during the dry period".

In order to reduce the frequency of intramammary injections of aqueous penicillin and obtain "a greater percentage of cures following a single injection", an oil in water emulsion was employed whereby penicillin in the aqueous phase was carried in a mineral oil medium (Foley, Stults, Lee and Bryne, 1949). In such an emulsion the rate of release of the penicillin was reduced, thus antibiotic levels were maintained in the udder for significantly longer periods than when the aqueous preparation was used, and "penicillin is more efficiently transported higher into the alveoli of the udder by negative geotropism than is the case when an aqueous vehicle is used".

The distributions of three antibacterial preperations, chlortetracycline ("Aureomycin"; Lederle Laboratories, now Cyanamid, Cyanamid House, Fareham Road, Gosport, Hampshire, PO13 OAS, England), oxytetracycline ("Terramycin"; Pfizer Ltd., Sandwich, Kent, CT13 9NJ, England), penicillin (Merck, now MSD Agvet, Hoddesdon, Hertfordshire, EN11 9BU, England) and streptomycin (Merck), were

compared after infusion into the bovine mammary gland as aqueous solutions and as "ointments" (Schipper, 1955); the constituents of the ointment were not recorded. "Antibiotics were administered in equal quantities" in either 500ml of sterile distilled water or 7.5g ointment per quarter. Post mortem examination of the udders showed that the aqueous solutions had a more uniform distribution throughout both infected and uninfected mammary glands. The ointment preparation often failed to penetrate to the dorsal region of the gland; this was especially marked in quarters with acute mastitis.

The distributions of aqueous penicillin and penicillin in oil infused into the udders of "normal" lactating goats (Ullberg, Hansson and Funke, 1958a) and cows and goats "with various types of mastitis" (Ullberg, Hansson and Funke, 1958b) was monitored using ^{35}S-labelled penicillin. The composition of the oil was described as "oleum arachidis, 5.0; aluminium monostearate, 250.0; Tween 80, 5.8". Both penicillin preparations showed a uniform distribution in the uninfected udders and reached all parts of the parenchyma although it was later stated that the aqueous penicillin was more concentrated in the ventral part of the udder whilst the penicillin in oil was found more in the dorsal area (Ullberg *et al*, 1958a); this was thought to be due to the lower "specific weight" (presumably specific gravity) of the oil. More penicillin was found in the parenchyma after infusions of the aqueous preparation whereas the oil based penicillin tended to remain in the milk ducts. There was no significant difference in the distribution seen at one hour after infusion and at eight hours after infusion. In unspecified diseased udders, both formulations had the same irregularity of penetration into the parts of the gland affected by pathological changes (Ullberg *et al*, 1958b). The distribution of the antibiotic was shown to be primarily dependent on passage through the milk ducts, with limited diffusion through the tissues. Pathological changes adversely influenced exposure to the penicillin of the more dorsal parts of a diseased gland. The advantages of the penicillin in oil preparation were that they had a longer retention time in the udder than the aqueous preparations, thus allowing a greater time interval between

successive infusions, and that they could be manufactured in advance in contrast to the aqueous penicillin preparations which, because of the instability of such preparations at that time, had to be made up immediately before being used (Ullberg *et al*, 1958a).

Penicillin, either in oil or as an aqueous solution, diffused through the dry udder in a similar way to that in the lactating udder, and penetration of the tissues was better in the non-lactating udder of cows and goats (Funke, 1961). No difference in the distribution of the antibiotic was seen between preparation using a mineral oil (78% liquid paraffin, 20% vaseline, 2% aluminium monostearate) and a vegetable oil (95.42% arachis oil, 4.46% aluminium monostearate, 0.12% Tween 80).

Little difference in the diffusion throughout the mammary glands was seen when 100,000 units of penicillin, either as sodium penicillin G, or the sodium, potassium or procaine salt, and in either an aqueous solution, an oil in water emulsion or a mineral or arachis oil base, were infused into 72 quarters of 24 cows which were slaughtered 24 to 96 hours after the infusion (Edwards, 1964). The concentration of penicillin in the various parts of the udders of cows slaughtered 24 hours after infusion showed little variation whichever medium was used, and residual levels of penicillin infused with the emulsion and the oil base carriers were present at 48 and 96 hours respectively after infusion. The inclusion of Tween in the oil bases assisted the dispersion of the penicillin. Concentration of penicillin was greater in the ventral part of the udder in cows that had been nearing the end of their lactation when the process of involution and shrinkage of ducts and alveoli had occurred. The greater amount of fibrous tissue in chronically infected quarters reduced the distribution and absorption of the antibiotic into the tissues, leading to higher concentrations of penicillin and longer retention times in the milk of those quarters.

Two quarters of each udder of 37 cows were infused with 200,000 units of both procaine penicillin G and dihydrostreptomycin ("Streptopen Veterinary Cerate"; Glaxo Laboratories Ltd., now Mallinckrodt Veterinary Ltd., Breakspear Road South, Harefield, Uxbridge, Middlesex, UB9 6LS, England) after the last milking of the lactation (Oliver *et al*, 1962), followed by all the teats being dipped in a mixed culture of *Staph. aureus* and *S. uberis*. Infection by the specific phage-type of staphylococci was prevented in all 74 infused quarters, whilst ten of the 37 uninfused control quarters became infected, nine with the specific phage-type of *Staph. aureus* used, and one with another strain. Two infused quarters became infected, one with *S. dysgalactiae* and one with *S. uberis*, although in the latter case it was impossible to tell whether it was the same strain as in the original culture.

Following the infusion of either one million units (599mg) or five million units (2993mg) (*sic*) of penicillin G in a quick release base or aqueous solution into six quarters of three cows after the last milking of the lactation (Smith, Neave and Jones, 1967), the concentration of penicillin in the udder secretion declined rapidly in the first 24 hours, and all the antibiotic had been destroyed or had diffused from the udder within five days of the infusion. From one hour after infusion, penicillin was detected in venous blood and was found in the non-infused quarters within two hours.

The therapeutic effects on two groups of cows receiving one of two dry cow intramammary preparations were evaluated against a group of control cows receiving no therapy (Smith, Westgarth, Jones, Neave, Dodd and Brander, 1967). The two preparations consisted of one gram of cloxacillin as the benzathine salt, and 0.2g of cloxacillin as the sodium salt, each in a three per cent aluminium monostearate solution in a mineral oil base; all infused cows also had their teats dipped in a hypochlorite solution (five per cent available chlorine). More than 50% of the 888 cows from 35 herds taking part in the trial had udder infections at the time of drying off and, with

a few exceptions, all the infections were caused by *Staph. aureus*, *S. agalactiae*, *S. dysgalactiae* or *S. uberis*. Of the 11% of quarters infected with *Staph. aureus* at drying off that were infused with benzathine cloxacillin, only two per cent were infected at calving of which only a very small proportion were new infections that developed during the dry period; similarly, of the 11% *Staph. aureus* infected quarters infused with sodium cloxacillin, just over five per cent remained infected, whilst staphylococcal infections in the untreated quarters rose from ten per cent to 14%. No individual figures were given for the effects of the two therapies on the streptococcal infections as both were reported to be equally good, the eight per cent of *S. agalactiae* quarter infections at drying off being virtually eliminated at calving time, the *S. dysgalactiae* quarter infections falling from three per cent to one half of one per cent, and the *S. uberis* infections falling from three per cent to two per cent although the authors suggested that more of the *S. uberis* infections existing at drying off were eliminated but new infections occurred during the dry period. In the untreated group, the levels of *S. agalactiae* and *S. dysgalactiae* infections during the dry period remained at seven per cent and three and a half per cent of quarters respectively, whilst *S. uberis* infections rose from two and a half per cent to five per cent of quarters.

The therapeutic value of a dry cow preparation containing one gram procaine penicillin G and 0.5g dihydrostreptomycin sulphate in a long-acting oily base was evaluated in a herd of 150 cows (Pugh, Harris, Marshall and Evans, 1973). At the end of lactation, 31 quarters were found to be infected with *S. agalactiae*, 21 with *S. uberis* and 12 with other streptococcal species. Four days post calving 62 of these 64 infected quarters were reported to be free of infection. The pathogens present in the two infected quarters were not reported. By 14 days post calving two quarters were found to be infected with *S. agalactiae* and three quarters with *S. uberis*. Thirty-nine out of 49 cases of *Staph. aureus* had similarly responded to the treatment during the dry period.

The therapeutic values of three dry cow preparations, 500mg benzathine cloxacillin ("Orbenin Dry Cow"; Beecham Laboratories Ltd., now Pfizer Ltd., Sandwich, Kent, CT13 9NJ, England), a combination of 250mg sodium novobiocin and 300,000iu penicillin ("Biotexin-PLA Cerate"; Glaxo Laboratories Ltd.) both preparations formulated in water repellent slow release bases, and 500mg spiramycin in a water miscible quick release base (May and Baker, now Rhône Mérieux Ltd., Spire Green Centre, Harlow, Essex, CM19 5TS, England) were compared using 2,312 quarters of 578 cows from ten farms (Loosmore, Howell, Adams, Barnett and Barr, 1968). A total of 458 quarters were found to be infected at the end of lactation, 288 with haemolytic *Staph. aureus*, 54 with *S. agalactiae*, 35 with *S. dysgalactiae*, and 81 with *S. uberis*. Fifty-two (2.8%) of the remaining 1,854 quarters developed new staphylococcal infections, 30 (1.6%) occurring during the dry period and 22 (1.2%) in the first month after calving. The number of new *S. agalactiae* and *S. dysgalactiae* infections was "too small to allow of analysis". When the quarters that had been infected before the dry period were sampled one month after calving, haemolytic staphylococci could not be isolated from 62% of the quarters treated with the benzathine cloxacillin and from 64% of the quarters treated with the combined sodium novobiocin and penicillin. However, only 40% of the quarters treated with spiramycin preparation were free of infection. No *S. agalactiae* and *S. dysgalactiae* infections continued into the next lactation except for one of the 25 cases of *S. agalactiae* infection which had received the spiramycin therapy, but *S. uberis* was still isolated from six (22%) of the 27 infections treated with cloxacillin, eight (28%) of the 29 infections treated with the sodium novobiocin and penicillin combination, and seven (28%) of the 25 infections treated with the spiramycin therapy.

The effectiveness of erythromycin (600mg in an oily solution, "Erytrotil Dry Cow"; Abbott Laboratories Ltd.) and benzathine cloxacillin (500mg in a slow release base, "Orbenin Intramammary Suspension Dry Cow"; Beecham Animal Health, now Pfizer

Ltd.) as dry cow preparations were compared in 109 cows (Clegg, Halliday and Hardie, 1975). Of the 26 quarters that were affected with *S. uberis* at the end of lactation, all 12 that were treated with erythromycin were free of infection after calving, whereas two of the 14 quarters treated with benzathine cloxacillin remained infected throughout the dry period. Of about 60 quarters in each treatment group infected with *Staph. aureus*, both preparations eliminated about 75% of the infections. No reference was made to infections caused by *S. agalactiae* and *S. dysgalactiae.*

The semi-synthetic cephalosporin cephalonium (250g) formulated in an oily base (Cepravin Dry Cow; Glaxo Laboratories Ltd.) was evaluated as a dry cow intramammary treatment in 396 quarters of 115 cows from seven herds (Curtis, Hendy, Watson, Harris, Davies and Marshall, 1977). Of the 19 quarters from which only *S. uberis* was isolated at the end of lactation, 17 were free of infection when sampled four days after calving, as were 34 of 57 quarters infected with *Staph. aureus*. In the ten quarters in which there was a mixed infection of *S. uberis* and *Staph. aureus*, seven were found to be free of both pathogens four days after calving. Ten quarters not infected at the end of lactation were found to be infected four days after calving, three with *S. uberis* and seven with *Staph. aureus*. From all 53 quarters infected with other streptococcal species at the end of lactation, no pathogens were isolated after the subsequent calving.

1.6.13 COMPARISON OF ROUTINE AND SELECTIVE DRY COW THERAPY

An in-herd comparison of routine and selective dry cow therapy was carried out using 740 cows from six herds over a period of four years (Robinson *et al*, 1983). Half the cows in each herd were given a comprehensive regime of one of three post milking teat dips, 0.5% w/w available iodine and 5.7% w/w glycerine BP ("Deosan teat dip"; Diversey, now Deosan Ltd., Weston Favell Centre, Northampton, NN3 4PD, England),

0.5% w/w available iodine and 5% w/w glycerine BP ("Iosan teat dip"; Ciba-Geigy Agrochemicals), or 0.5% w/w available iodine and 10% w/w glycerine BP ("Iosan Superdip"; Ciba-Geigy Agrochemicals), and post-lactation dry cow intramammary therapy of either one gram procaine penicillin and 0.5g dihydrostreptomycin ("Streptopen Dry Cow"; Glaxovet, now Pitman-Moore Ltd.), or 500mg cloxacillin ("Orbenin Dry Cow"; Beecham Animal Health). The remaining cows in each herd did not have their teats dipped and were given a "partial treatment" of dry cow therapy based on bacteriological examination of "the drying off quarter milk samples", which were usually collected by the herdsmen and examined at the practice laboratory. As it would take at least two days to get a usable bacteriological analysis and antibacterial sensitivity in order to select the correct dry cow therapy it has to be presumed that either "drying off milk samples" were taken several days before the termination of lactation or that there was a delay of at least two days between the end of lactation and the infusion of dry cow therapy. Throughout the study, *S. uberis* was the commonest pathogen isolated from the clinical mastitis cases, with 111 infections (26 cases per 100 cows per year) in the partial treatment half herds and 66 infections (16 cases per 100 cows per year) in the half herds receiving the comprehensive therapy, however the authors suggested this was due largely to the high incidence of *S. uberis* infection on one farm. Conversely, the average incidence rate of the other major environmental mastitis pathogen, *E. coli*, was higher in the full treatment half herds than the partial treatment half herds (Robinson *et al*, 1983). After a further two years of the trial, the incidence of *S. uberis* mastitis in the partial treatment half herds was 72% higher than in the full treatment half herds, whilst coliform infection was 18% lower in the partial treatment half herds than in the full treatment half herds (Robinson *et al*, 1985).

1.6.14 OTHER ANTIBACTERIAL AGENTS USED IN MASTITIS TREATMENT

1.6.14a NEOMYCIN

The therapeutic value of neomycin sulphate in the treatment of bovine mastitis was investigated in 284 infected quarters of 128 cows in 27 herds (Tucker and Johnson, 1953). Vials of 500mg neomycin sulphate were mixed in 3.5g of a water miscible base ("Teatube"; Upjohn, Kalamazoo, Michigan, USA), or in either ten or 20ml of a water-in-oil vehicle (Heydon Chemical Corporation, Princeton, New Jersey, USA). Each quarter was given either one or two infusions, the latter with an interval of 48 hours between infusions. Milk samples from treated quarters were collected two and four weeks after treatment; if the samples were visibly normal and no pathogen could be isolated from them, the quarters were deemed to be free of infection. The recovery rate was not significantly affected by the frequency of infusion, the vehicle used, or the volume of infusion; 29 (27%) of 108 quarters with *S. agalactiae* infection responded to treatment as did 26 (41%) of 64 quarters with streptococcal infection other than those caused by *S. agalactiae*, and 14 (25%) of 56 quarters with haemolytic staphylococcal infection.

The response to two infusions of 500mg of neomycin sulphate in 3.5g ointment base ("Teatube"; Upjohn), the infusions being given either 24 or 48 hours apart, was assessed in 40 infected quarters from 20 cows (Aynsley and Hughes, 1954). The infections varied from subclinical to acute clinical mastitis but no further details were given. Success of the treatment was based on the bacterial examination of four or more post treatment milk samples collected weekly. Eight (67%) of 12 *S. dysgalactiae* infections responded to the treatment but only four (36%) of 11 *Staph. pyogenes* infections and four (31%) of 13 *S. uberis* infections responded similarly; all four cases

from which *C. pyogenes* (*A. pyogenes*) had been isolated failed to respond to the treatment. No comment was made on the relative value of 24 and 48 hour intervals between treatments, and the effect of neomycin on other notable pathogens was not included because "it has been amply demonstrated that infections with *S. agalactiae* respond well to penicillin and as yet infections with gram negative bacteria are not an important feature in this country (Great Britain)".

1.6.14b OXYTETRACYCLINE

Oxytetracycline therapy was assessed in cases of mastitis in the 120 cows of the Iowa State College dairy herd, which had been free of *S. agalactiae* for the previous 11 years (Barnes, 1955). Treatment of clinical and subclinical cases of mastitis consisted of three daily infusions of "one 400mg tube of commercial ointment infusion (7.7Gm, 60mg/Gm) of oxytetracycline". The dose was doubled and treatment prolonged for "severe cases". Post-treatment "periodic" bacterial examination of milk samples showed that nine (75%) of 12 clinical cases and 29 (85%) of 34 subclinical cases of *S. uberis* recovered with the treatment. Only one (33%) of three clinical cases of *S. dysgalactiae* (there were no subclinical cases), and 11 (25%) of 44 clinical and subclinical cases of *Staph. aureus* were successfully treated, but the drug had a better effect on coliform mastitis with a 70% recovery. The intramammary infusion of oxytetracycline into the udder produced a measurable amount of local inflammation as measured by the catalase and Whiteside test and manual palpation. Intra-muscular injections of oxytetracycline (one to two grams oxytetracycline repeated at 12 or 24 hour intervals) were routinely found to relieve the systemic signs which accompanied acute mastitis, irrespective of the aetiological agent.

1.6.14c ERYTHROMYCIN

Of 245 cows in four herds, 52 cases of subclinical *Staph. aureus* mastitis were treated with three intramammary infusions of 300mg erythromycin (no details of the vehicle were given) at 12 or 24 hour intervals (Schultz, 1968) and, in 39 of the cases, the pathogen could not be isolated from milk samples collected one and three weeks after treatment; the results showed little difference whether a 12 or 24 hour treatment interval was used. All 17 cases of subclinical *S. agalactiae* mastitis responded successfully to the same treatment with a 12 hour treatment interval, as did six of seven cases of subclinical *S. uberis* mastitis; however *S. uberis* isolates were identified solely on their ability to hydrolyse aesculin. Of clinical cases on a 12 hour treatment interval, eight of ten cases of *Staph. aureus* mastitis recovered, as did six of eight cases of *S. agalactiae* mastitis and both cases of *S. uberis* mastitis; no clinical cases were treated on a 24 hour interval basis.

1.6.14d CEPHALOSPORINS

The cephalosporin, cephoxazole (250mg as the sodium salt) in combination with 250mg procaine penicillin G in a quick release base ("Cepoxillin"; Glaxo) was investigated as a treatment for bovine mastitis (Harris, Davies, Marshall, Evans, Hendy and Watson, 1977). The response was assessed by clinical examination of 174 treated cases and, in 167 of the 174 cases, by bacterial examination of milk samples collected seven and 14 days after treatment; the lack of examination of milk samples from the other seven cases is not explained. A successful bacteriological response was claimed when there was either no evidence of the pathogen or a reduction of at least 100 fold in the concentration of colony forming units isolated from the post-treatment milk samples. Twenty-five (71%) of 35 quarters infected with *S. uberis* showed a successful "bacteriological response", and there was a "clinical response" in a further four quarters

in which the inflammation had disappeared and milk yield and quality had returned to normal although the pathogen had not been eliminated. Of other cases from which only one pathogen was isolated, there was a successful "bacteriological response" in all 14 quarters infected with *S. agalactiae*, in 11 (92%) of 12 quarters infected with *S. dysgalactiae*, and in nine (82%) of 11 quarters infected with *E. coli*, but in only three (30%) of ten quarters infected with *Staph. aureus.*

1.6.15 EFFECTS OF ANTIBACTERIAL THERAPY ON THE PREVALENCE OF PATHOGENIC SPECIES

Plommet and le Louedec (1975) noted that *S. agalactiae*, which could not exist outside the mammary gland and was susceptible to penicillin, had been "replaced" in the udder by pathogens more able to resist penicillin such as *Staph. aureus*, or by pathogens which, because they could exist in the cows' environment outside the mammary gland, were more difficult to eliminate from a herd, such as *S. uberis*, *E. coli* and *Ps. aeruginosa*. Eradication of *S. agalactiae* required treatment of all quarters with penicillin (two intramammary infusions of 100,000 units at 48 hours interval), as a single infected quarter could recontaminate a herd very quickly. However, this treatment had to be accompanied by a high level of milking hygiene if the *S. agalactiae* was not to be replaced by *Staph. aureus* or other streptococci otherwise "the eradication of *S. agalactiae* is a somewhat academic satisfaction".

In a review of mastitis control measures, Smith (1983) stated that mastitis control procedures of dry cow therapy and post-milking teat disinfection used against *S. agalactiae* and *Staph. aureus* did not effectively control infection caused by mastitis pathogens which could also live outside the mammary gland, the so-called environmental mastitis pathogens .

1.6.16 INTRAMAMMARY DEVICES

An intramammary device was designed to be inserted through the teat canal into the teat cistern by a canula in an attempt to raise the level of leucocytes and provide local protection against intramammary infection, especially those caused by environmental pathogens (Paape, Ziv and Schultze, 1988). The first intramammary device was made of extruded polyethylene 2.5mm in diameter and 115mm long, smooth over its entire surface and moulded so as to take the form of a loop 25mm in diameter once it had been inserted into the teat cistern. This loop did not sufficiently elevate the somatic cell count to prevent infection and the large bore diameter made it difficult to insert through the teat canal, especially in first calf heifers.

By reducing the bore diameter to two millimetres (in later designs to 1.5mm) and abrading the polyethylene surface with emery paper (Paape, Schultze, Cortlett and Weinland, 1988), the average somatic cell count of the milk from the five quarters of five cows carrying the loop rose from 700,000 cells per ml at milking to 1.17 million cells per ml six hours after milking, dropping to 850,000 cells per ml 11 hours after milking. This compared with levels of 430,000, 640,000 (four hours after milking) and 260,000 cells per ml respectively for the milk from another five quarters of the same cows carrying smooth loops of the same dimensions, and 150,000 to 240,000 cells per ml for the first eight hours after milking rising to 600,000 cells per ml 11 hours after milking for the milk from the remaining nine control quarters of the same cows in which no loop had been inserted; one control quarter had become infected with a coagulase negative staphylococcus and took no further part in the experiment. Two months after the insertion of the loops, the nineteen quarters were inoculated with 250 colony forming units of *S. uberis*. This resulted in two of the five quarters with the abraded loop, four of the five quarters with the smooth loop, and eight of the nine control quarters becoming infected as determined by bacterial analysis of foremilk collected individually

from all 19 quarters for the ten milkings following the inoculation of *S. uberis*. It was suggested that the mechanism of the immunity was due to the release of neutrophil chemotatic factors by macrophages which had adhered to the nylon surface, this being reflected in an increase in the milk somatic cell count.

However, within a year of implantation, the somatic cell count had dropped and the incidence of infection had risen, which correlated with the abraded nylon having become coated with an amorphous material (Paape, Ziv and Schultze, 1988). Coating the device with sulphur hexafluoride appeared to prevent the build up of amorphous material, and in field trials, 2,182 cases of clinical mastitis were recorded in non-implanted control cows but only 475 cases in the cows with the modified implant; the implanted cows also showed an increase of 1.9kg milk per day over the non-implanted control cows. However, the implants caused tissue irritability to the epithelial lining of the gland cistern leading to pin point haemorrhages which resulted in a significant increase in the concentration of red blood cells in milk; from a normal red blood cell count of 26,000 cells per ml in milk from a non-implanted udder, a smooth implant caused a rise to over 11 million cells per ml, and an abraded implant a rise to over 27 million cells per ml.

1.6.17 AN EARLY TRIAL IN PASSIVE IMMUNITY

The serous discharge, collected from a quarter that had been experimentally infected with *B. lactis aerogenes* through the teat duct, had "an inhibitory effect on the development of the bacteria, for although (the discharge was) kept in the incubator for days no increases whatever in the organism could be demonstrated" (Gilruth and MacDonald, 1911). Twelve days after the quarter was infected, 50ml of blood serum was drawn from the same cow and injected subcutaneously into another cow which had been similarly infected via the teat canal three days earlier. The second cow showed a

rapid recovery, with the udder being of more or less normal appearance after two days, and the milk showing no abnormalities after a further six days.

1.6.18 VACCINATION

Intradermal inoculations of killed staphylococci and cell wall components showed that cows possessed both an immediate antibody complement dependent inflammatory response and a delayed cellular dependent hypersensitivity reaction, cows with a history of staphylococcal mastitis producing a faster response (Reiter and Bramley, 1975). Intradermal inoculation of large numbers (at least one million colony forming units) of live *Staph. aureus* organisms were needed to produce an intradermal lesion, the severity of which corresponded to the intramammary virulence of the bacterial strain. However, the intramammary and parenteral staphylococcal vaccination programme failed to protect the majority of udders when challenged with the infusion of "small numbers" of staphylococci.

In contrast, there has been some limited success in vaccination against streptococcal mastitis (Norcross, 1970; Norcross and Stark, 1970). Each streptococcal species could be divided into antigenic types, *S. agalactiae* having at least five types differentiated serologically by a carbohydrate antigen (Wilkinson and Moody, 1969). Vaccination against one type protected animals from infection of that type, but did not protect against other types of the same species (Norcross, Dodd and Stark, 1968). As there is a large number of phage types of staphylococci (Norcross and Stark, 1970) and strains of *S. uberis* (Hill and Leigh, 1989), the possibility of cross protection from these species could be more difficult to achieve.

Whilst the Lancefield group B carbohydrate antigen of *S. agalactiae* was efficient in stimulating antibody production, the antibodies were not protective and did not confer

immunity (Norcross, 1970): With *S. uberis*, even if the Lancefield group antigens did stimulate antibody production, only about 20% of *S. uberis* isolates possess such an antigen, predominantly group E (Cullen 1967). Furthermore, 15 strains of the Edwards group III (*S. uberis*) were classified into three subtypes identified on the basis of agglutination serological typing (Stableforth, 1932), and 136 *S. uberis* isolates were similarly divided into 11 serological types (Plastridge and Williams, 1939).

The development of the possible use of vaccination for mastitis control has continued. Infusions of 1,000 colony forming units of *S. uberis* into one quarter of each of 11 cows and two quarters in six cows caused 19 of the 23 quarters to become infected within two to five days and one to become infected after 57 days (Hill, 1988). Two of the 11 single quarters and one of one of the six pairs of quarters remained uninfected. After successful treatment of the resultant clinical infections, two quarters of each cow were reinfected between 27 and 105 days later, the original quarter and one other in the 11 cows previously infected in only one quarter, and either the same two quarters or one of the original pair and one other in the other six cows. Only 11 of the 34 quarters became infected, five for the first time and six for the second time. To explain the resistance by three quarters to the first challenge, it was reported that the one cow which resisted both first and second challenges had suffered a naturally induced *S. uberis* infection earlier in the same lactation, and for the other two cows it was suggested that immunity may have been stimulated either by undetected subclinical mastitis or *S. uberis* inhabiting another part of the body, for example the gastro-intestinal or the genital tract, as found by Kruze and Bramley (1982).

1.7 MILK SOMATIC CELL COUNTS

According to Doane (1905), Stokes (no date or reference given) was the first to note the presence of leucocytes in milk and to devise a method for estimating the comparative

number present: Ten millilitres of milk were centrifuged, the supernatant discarded and a loopful of the precipitated sediment smeared on a coverslip, stained with methylene blue and viewed with a microscope "using the 1-12 objective"; Campbell (1909) reported that Stokes used a 1/12 oil-immersion lens.

The lowest number of cells per field considered to be indicative of an uninfected milk sample was a matter of individual preference. Doane (1905) reported that Stokes regarded the presence of any leucocytes to be evidence of an abnormal state. However, the large number of cows with leucocytes in their milk was too great to think of excluding them from "furnishing milk for consumption", so the limit was set at five leucocytes per field: "This eliminated a few cows, not enough under the best conditions to cause too much objection, and the work to be discredited". The same author reported that Stokes subsequently revised the limit up to 25 cells per field without giving any reason, and that Bergey (1904) had taken an upper limit of ten leucocytes per field to indicate infection, "while Eastes (no date or reference given) simply said a large number, leaving himself plenty of room". No finite number of leucocytes per unit volume of milk could be calculated by Stokes' method as no account was taken of the volume of sediment left after centrifugation, and a loopful of sediment was an indefinite measure on which to base calculations of concentration (Doane, 1905).

Modifications to Stokes' method were made by Doane and Buckley (Doane, 1905) which allowed a more accurate measurement of the leucocyte concentration. The cream that had collected on the surface of the milk after the centrifugation was removed with a swab and the supernatant was siphoned off. The remaining sediment was stained with methylene blue and then thoroughly mixed with distilled water to make a volume of one millilitre. This rendered the stain less dense, and gave an arbitrary volume from which to calculate the cell concentration. A drop of the leucocyte solution was placed in an un-named blood counter holding "one-tenth cubic millimetre", and the cells counted

using a microscope; later Campbell (1909) stated that a Thoma-Zeiss blood counting apparatus had been used. Doane (1905) found that no milk sample was ever free of leucocytes from which he concluded that the cells were a normal constituent of milk: Of 199 udder or quarter milk samples, from two herds "looked after with more than ordinary care" and therefore presumed to give milk "as pure as from the great majority of herds", 131 samples had a leucocyte count of less than 100,000 cells per ml, 60 samples had a count of between 100,000 and one million cells per ml, and eight samples had a count of over one million cells per ml. Milk of abnormal appearance or from an inflamed quarter was always found to contain "an abnormally large number of leucocytes". In addition, milk collected individually from the four quarters of an udder had different leucocyte counts, and on successive days, the count from any quarter could vary by a factor of over 100, however the variation was usually within a factor of three; it was not stated whether the samples were drawn at the same stage of the milking process.

Using the Doane-Buckley method, the numbers of leucocytes, recovered from portions of 49 samples of milk that had been heated at 70°C, were increased by between 0.2 and 300% compared with leucocyte numbers from portions of the same milk samples that had not been heated (Russell and Hoffman, 1908): Heated portions from another nine samples showed a decreased leucocyte count of between 0.2 and 71.6%. When portions of 31 milk samples were heated to between 50° and 80°C, it was found that the greatest variation in the leucocyte count occurred in those portions that had been heated to a temperature of between 60° to 70°C. The authors concluded that the heat broke up the fat globules sufficiently to prevent the leucocytes being caught up in the fat layer, so that they were precipitated during the subsequent centrifugation and hence able to be counted, instead of being removed with the cream. Campbell (1909) agreed that the optimum temperature for heating the milk was between 60° and 70°C; in 40 out of 42 samples, heating the milk increased the cell count by a factor of between 12 and

1,360. Cooling the milk followed by a second heating did not further increase the cell count.

Campbell (1909) made modifications to the Doane-Buckley method to minimise the fat globules in the final precipitate, and to avoid the use of a stain that precipitated in the blood-counting chamber. After the first centrifugation of the milk, the fat layer and supernatant liquid were aspirated off until five to six millilitres remained. This was topped up to the ten millilitre mark with distilled water and re-centrifuged; the procedure was repeated several times until the supernatant cleared. The supernatant was then siphoned off until one millilitre of sediment and supernatant remained; this was thoroughly mixed before being put in a blood-counting apparatus.

According to Doane (1905), Bergey (1904) found that leucocyte numbers increased with the presence of bacteria in the milk but sometimes high leucocyte counts occurred in the absence of bacteria, and low leucocyte counts were found when bacteria were present; Doane stressed the importance of either examining the milk immediately after collection or freezing it for later examination, thus preventing bacteria already present in the milk or contaminant bacteria multiplying. The lack of a consistent, direct relationship between high leucocyte numbers and the presence of bacteria in bovine milk was more recently demonstrated in clinical cases of mastitis caused by *Staph. aureus* infection (Torgerson, Gibbs and Anderson, 1992).

The concentration of leucocytes in milk samples taken from "the middle milk" was always less than the concentration in the foremilk and the strippings (Savage, 1908). In addition, the leucocyte count rose towards the end of lactation, concurrent with the decline in milk yield, and was also raised at parturition but dropped significantly within one week. The cell count was also greatly increased when mastitis was present, and

could remain elevated for several months following the infection, even though the milk had regained a normal appearance.

A milk somatic cell count of 70,000 cells per ml or less was considered to be "normal" by Blackburn (1956). In cases of staphylococcal or streptococcal mastitis, where there was a total cell count of over 200,000 cells per ml of milk, the percentage of neutrophils was between 65 and 95% of the total cells. Following successful treatment of staphylococcal or streptococcal infection, up to five weeks could elapse before the cell count returned to pre-infection levels, although occasionally the cell count dropped to "normal" within one week. Even when the fall in the cell count was protracted, there was still a marked drop in the percentage of neutrophils in the milk to less than 30% within one week of successful treatment. The count of some treated quarters, however, never returned to below 70,000 cells per ml, and the cell profile resembled that normally seen in late lactation, with neutrophils accounting for less than 30%, the remainder being cast epithelial cells. Unless reinfection occurred, the majority of quarters would have a "normal" cell count at the start of the next lactation. To explain why some quarters retained a raised cell count with a high percentage of neutrophils after the elimination of the infectious agent, it was suggested that, as those particular quarters always gave a reduced yield for the remainder of that lactation in comparison with the other quarters of the udder, the post-infected quarters would be milked out quicker and hence suffer trauma from being overmilked, thus maintaining the high percentage of neutrophils.

The incidence of subclinical mastitis caused by coagulase-positive staphylococci was reduced from 25% to 5% by "extensive treatment" (not detailed) before the start of a survey in 1953 at the Hannah Dairy Research Institute, Ayrshire, Scotland (Blackburn, 1966). During the following 12 years, approximately 38,000 foremilk samples were collected from the cows at the Institute. The average cell count was found to increase in

succeeding lactations, 300,000 cells per ml in the first lactation, 510,000 cells per ml in the second, and then rising steadily to 1.08 million cells per ml by the seventh lactation. The rise was almost wholly due to an increase in the number of neutrophils, the counts of cells other than neutrophils remaining constant except for a small rise between the first and second lactation; the identity of the cells other than neutrophils was never defined. For more than 12 months in the seventh and eighth year of the survey, a time when no coagulase-positive staphylococci could be isolated, the average total cell count of all milk samples was 300,000 cells per ml, which also happened to be the average cell count for the cows in their first lactation. However, the percentage of neutrophils in the milk of the first lactation cows was 50% compared with 80% for the rest of the herd, from which it was concluded that, with successive lactations, the mastitis lesions in the udder lessened in extent, based on the low numbers of epithelial cells, but were of greater severity, as reflected by the increased presence of neutrophils (Blackburn, 1966).

In a further report of the Hannah study (Blackburn, 1968), the average somatic cell count of milk samples, from which no staphylococci, streptococci or coliforms were isolated, increased from 190,000 cells per ml for cows in their first lactation to 600,000 cells per ml for the seventh lactation, the vast majority of the increase being due solely to an increase in neutrophils. Infected samples showed an increase in both neutrophils and other cells. The herd used for this study was reported to be free of *S. agalactiae*; the other species of streptococci present were not identified but most hydrolysed aesculin. The average cell count for samples from which streptococci other than *S. agalactiae* were isolated over the first seven lactations was 241,000 cells per ml of which 69% were neutrophils. The cell counts varied with the lactation, the average total cell count for the first lactation being 148,000 cells per ml (80% neutrophils); this peaked in the next lactation at 462,000 cells per ml (75% neutrophils), and in the following five lactations the total cell counts were between 167,000 cells per ml (69% neutrophils) and 314,000 cells per ml (65% neutrophils).

The Milk Marketing Boards of the United Kingdom began to use bulk milk somatic cell counts in 1971 to monitor the prevalence of subclinical mastitis, this service being available to the farmer for a fee (Booth, 1991; 1994). Since 1977, every herd has been monitored every month on a free advisory basis (Booth, 1988; 1994). In the first 16 years of monitoring the average milk somatic cell count showed a stepped decline with three plateaux being separated by a steep fall (Booth, 1988). From 1971 to 1974, the cell count averaged 568,000 cells per ml followed by a drop of 90,000 cells per ml over the next two years that Booth (1988) credited to a high inflation rate and severe pressure on financial returns forcing nearly 10,000 dairy farms out of milk production in the years 1974 to 1976; as these herds had averaged 50,000 to 100,000 cells per ml above the national average their loss helped to reduce the national milk somatic cell count. The reduction in cell count was also helped by favourably high cull cow prices at a time of 18 months low rainfall that precipitated a feed shortage thus decreasing the national herd by 150,000 cows. From 1975 to 1982, the national somatic cell count averaged 478,000 cells per ml. The second significant fall in cell counts occurred in 1983 following the introduction of a bonus payment for a low total bacterial cell count which, with the introduction of milk quotas in the same year, led to many unproductive and mastitis prone cows being culled, with the result that somatic cell counts averaged 375,000 cells per ml for the years 1983 to 1986. Between 1986 and 1991, the cell count fell from 352,000 to 310,000 cells per ml in England and Wales (Booth, 1993). In 1992, the method of calculating the cell count for each farm was changed so that the count became the geometric mean of the previous three months' weekly cell counts and, using the revised method of calculation, the somatic cell count for 1992 was 282,000 cells per ml.

In the United Kingdom, the system of bonus payments and penalties based on bulk milk somatic cell counts began in October 1991, different scales being set by the Milk Marketing Boards of England and Wales, and those for Scotland.

Band	Somatic Cell Count (1,000 cells per ml)	Bonus(+) / Penalty(-) Payment (pence per litre)
1	000-400	+ 0.2
2	401-700	0
3	701-1,000	- 0.2
4	over 1,000	- 0.4

Table 1.1 England and Wales Milk Marketing Board milk somatic cell count penalty/bonus payments, 1991 to 1993

The scheme for Scotland, with a less generous bonus payment and more severe penalties was introduced in two stages, the bonus payments began in April 1991 whilst the schedule of penalties began at the start of 1992.

Band	Somatic Cell Count (1,000 cells per ml)	Bonus(+) / Penalty(-) Payment (pence per litre)
1	000-400	+ 0.1
2	401-600	- 0.2
3	over 600	- 0.4

Table 1.2 Scottish Milk Marketing Board milk somatic cell count penalty/bonus payments in Scotland, 1991 to 1993

In 1993 the schedule the bonus for a low somatic cell count was discontinued and the schedule of penalties revised.

Band	Somatic Cell Count (1,000 cells per ml)	Bonus(+) / Penalty(-) Payment (pence per litre)
1	000-400	0
2	401-500	- 0.5
3	501-1,000	- 1.0
4	over 1,000	- 2.0

Table 1.3 England and Wales Milk Marketing Board milk somatic cell count penalty/bonus payments, 1993 onward (Booth, 1993)

Herds with an annual average cell count of less than 400,000 cells per ml produced 85% of the milk supply (Booth, 1994), however only 40% of herds had an annual average cell count of less than 250,000 cells per ml.

Milk quality is also measured by means of monitoring antibiotic residues and total bacterial count in milk. Measurement of antibiotic residues with financial penalties for failures was first instigated by the Milk Marketing Boards in 1966; since then the sensitivity of the testing has greatly increased, as have the financial penalties (Booth, 1994). Total bacterial counts were introduced in 1982 following 30 years of measuring the bacterial content of milk by the resazurin test (Booth, 1994). Four levels of contamination were recognised and classed as bands A, B, C and D. Band D was amalgamated with band C in 1987.

Band	Total Bacteria Count (cells per ml)
A	20,000 or less
B	21,000 - 100,000
C	100,000 - 250,000 (since 1987 - over 100,000)
D	over 250,000

Table 1.4 England and Wales Milk Marketing Board total bacterial count bands (Booth, 1994)

In the ten years from 1983/84 to 1993/94, the proportion of herds in band A rose from 65% to over 86%, whilst herds in band B decreased from nearly 31% to under 14%, and those in band C (and D) from just over four per cent to 0.1% (Booth, 1994); in 1994, over 92% of all milk was of band A quality.

The European Community Council directive 92/46 (Milk Hygiene) required that, from 9th May 1995, milk for liquid consumption should have a total bacterial cell count of no more than 100,000 cells per ml and a somatic cell count of no more than 400,000 cells per ml. Under the same directive, milk for manufacturing and processing should have a total bacterial cell count of no more than 400,000 cells per ml and a somatic cell count of no more than 500,000 cells per ml (Madders, 1995). On 1st January 1998, the total bacterial cell count and the somatic cell count of milk for manufacturing and processing will be the same as that of milk for consumption. At present, no individual sample of each farm's milk is collected, the only sample being taken when the tanker lorry reaches the milk depot where a composite sample is taken of all the farms' milk collected on that round, thus milk of poor hygienic quality from some farms can be masked by having been mixed with better quality milk from other farms. This will change on 1st July 1997 when every farm's milk will be sampled individually before being collected.

CHAPTER TWO

MATERIALS AND METHODS

2.1 INTRODUCTION

The widespread application of mastitis control measures based on post milking teat disinfection and dry cow therapy has led to a reduction in the incidence of clinical and subclinical mastitis (Bramley, 1984). However, these control measures have been of limited effectiveness against the so called 'environmental' pathogens, that is pathogens that can live outside the bovine udder, most notably *S. uberis* and the coliform bacteria.

Many strains of *S. uberis* have been identified by various techniques including the DNA typing (fingerprinting) method of Hill and Leigh (1989), and recent evidence has shown that the species may, in fact, be two distinct species, *S. uberis* and *S. parauberis* (Williams and Collins, 1990).

The purpose of the study was to carry out an epidemiological survey of *S. uberis*, using a commercial dairy herd in which *S. uberis* was endemic, by regular sampling of the cows' milk, skin, vaginas, rectums and bedding, and typing the different strains of the pathogen isolated. Monitoring the isolates over a period of time would show whether all the isolated strains of *S. uberis* could be pathogenic, or whether only some strains were pathogenic whilst others were merely harmless commensals living outside the cows' mammary gland; it was also anticipated that the relative roles of *S. uberis* and *S. parauberis* could be investigated in relation to habitat and pathogenicity.

In addition, using the cultural characteristics of known isolates of *S. uberis*, the formidable array of biochemical tests normally used to identify *S. uberis* was to be reduced by devising a protocol for the rapid identification of *S. uberis* using as few isolation and identification techniques consistent with accurate identification. Other work undertaken during the study included a comparison of the *S. uberis* enrichment ability of three nutrient broths, and the assessment of the number of colony forming units in a single colony of *S. uberis* grown on blood agar. DNA fingerprinting techniques were also used to show the different restriction fragment patterns of the various *S. uberis* isolates acquired.

On the occasions when other mastitis pathogens, predominantly *Staph. aureus*, were isolated, antimicrobial susceptibility tests were carried out on a representative sample of the pathogens.

2.2 ON FARM PROTOCOL

2.2.1 ACCESS TO FARMS

In order to obtain access to commercial dairy herds for the study of *S. uberis* infection, a number of farm animal veterinary practices in Central, West and South West Scotland were contacted. With the consent of the farmers concerned, practices referred herds that were experiencing one or more of the following; clinical cases of *S. uberis* mastitis, an ongoing mastitis problem of unknown aetiology, or a bulk tank milk somatic cell count of more than 400,000 cells per ml of milk, which was high enough to invoke a penalty in the payment made to the farmer for his milk by the Scottish Milk Marketing Board.

Initial contact with the farmers was made by telephone to explain the nature of the study and make sure of their willingness to cooperate, as well as to obtain general details

about the farm (farm size, herd size, mastitis problem etc.). This was often followed by a preliminary visit to the farm.

2.2.2 MILK SOMATIC CELL COUNTS

Following the initial contact, the farms that used the Scottish Milk Records Association (SMRA) Cell Count Advisory Service supplied details of the previous months' milk somatic cell counts.

Where possible, the consecutive monthly milk somatic cell count records for each cow were collated to show the changes of cell count over the period of the records. The trend of each cow's cell count was enhanced by the use of 'highlighting' fibre tipped pens of various colours. All milk somatic cell counts of over 600,000 cells per ml were highlighted in pink, those of 400,000 to 600,000 cells per ml were highlighted in orange, and those of 250,000 to 400,000 cells per ml were highlighted in yellow. Milk somatic cell counts of less than 250,000 cells per ml were left unhighlighted.

A provisional list of cows to be sampled usually included all those recorded at the most recent SMRA visit with a milk somatic cell count of over 250,000 cells per ml. However, cows with a milk somatic cell count of less than 250,000 cells per ml were sometimes also selected to determine whether *S. uberis* could be isolated from the milk of udders showing no evidence of mastitis.

One farm did not participate in the SMRA milk somatic cell count scheme. A portion of every milk sample collected from that farm was sent to the Haematology Laboratory of the Department of Veterinary Pathology, Glasgow University Veterinary School, for the somatic cells to be counted using a Coulter counter model ZF (Coulter Electronics, Luton, Bedfordshire, England).

2.2.3 ON FARM TECHNIQUES

Farm visits, for the purpose of collecting samples, were arranged to coincide with the afternoon milking, enough time being allowed before milking started to obtain further information on the herd and its management. As a result of the initial contact and subsequent conversation at the time of the sampling visit, a comprehensive picture of the farm in general and the herd management in detail was obtained and recorded on a Farm Information Sheet (appendix II).

The list of the cows to be sampled and the sampling procedure was discussed with the farmer at the sampling visit and augmented according to the current circumstances, for instance, cows on the provisional sampling list that had recently been dried off at the end of their lactation, or had been receiving antibacterial therapy in the last four weeks, were removed from the sampling list, and other cows with a history of recurrent clinical mastitis were sometimes added to the list.

The number of samples that would be generated from all the cows on the sampling list sometimes exceeded the number of samples that could be put through the particular isolation protocol being used at that stage of the study and, unless a specific cow was especially required for sampling, the first cows on the list that entered the parlour up to the required number, were the cows that were sampled.

2.2.4 COLLECTION OF MILK SAMPLES

Milk samples were collected either individually from each quarter or as a composite sample from the milking machine collecting jar. Quarter samples were taken before milking but following the farm's normal pre-milking udder and teat cleaning routine. The foremilk was drawn from each teat after which a sample of milk was collected into a

sterile 30ml glass universal container, which was tilted as much towards the horizontal as possible so as to exclude contamination falling into the mouth of the container. Care was taken to avoid the tip of the teat being touched either by hand or the lip of the container. If possible, at least ten millilitres of milk were drawn from each quarter although, with fractious cows, this was not always possible. Composite milk samples were drawn from the milk collecting jar into a sterile 30ml glass universal container after each cow had been milked. Before the sample was collected from the jar tap, milk was allowed to run freely from the tap for a few seconds to minimise any contamination of the sample from any milk from the previous cow left in the tap or the bottom of the jar.

Once filled, the universal containers were tightly sealed and labelled, using indelible ink, with the cow's number and, where applicable, the quarter. The cows on five farms were identified by freeze brand numbers on their rump, one farm used numbered collars and one farm used large numbered ear tags.

2.2.5 COLLECTION OF RECTAL AND VAGINAL SWABS

Rectal and vaginal swabs were collected using dry plain sterile cotton wool swabs (Medical Wire and Equipment Co. [Bath] Ltd., Corsham, Wiltshire, SN13 9RT, England), care being taken to exclude any external contamination; the entrance to the orifice was opened manually before inserting the swab into the first five to ten centimetres of the passage, rotating the swab and then withdrawing it, and replacing it in its container.

2.2.6 COLLECTION OF UDDER SKIN SWABS

Udder skin was sampled by rubbing cotton wool swabs (Medical Wire and Equipment Co. [Bath] Ltd.), previously moistened with Todd Hewitt broth (Oxoid), on the skin of

the lateral udder wall, on the side nearest to the operator. Skin swabs were replaced in their individual protective container in which enough Todd Hewitt broth had been placed to cover the swab.

2.2.7 STORAGE OF SAMPLES DURING TRANSPORTATION AND AT THE LABORATORY

Once the last sample had been collected, all the samples were transported to the laboratory packed in a closed insulated box containing ice packs. At the laboratory, all samples were stored at 4°C until the initial plating out and/or inoculation into broth, which was completed within six hours of sampling. The samples were retained at 4°C for another two days for further examination, if necessary.

2.3 LABORATORY TECHNIQUES FOR THE ISOLATION AND IDENTIFICATION OF *STREPTOCOCCUS UBERIS*

2.3.1 BLOOD AGAR MEDIA

Blood agar and Edwards agar media were prepared from Blood Agar Base No 2 (Oxoid) and Edwards Medium (Oxoid) respectively. During the first part of the study, the five per cent (v/v) blood component used in the media was defibrinated horse blood (Becton Dickinson UK Ltd., Between Towns Road, Cowley, Oxford, OX4 3LY, England), but this was later replaced by defibrinated sheep blood (Becton Dickinson) as a result of a change in laboratory purchasing policy.

After incubation on blood agar at 37°C for 24 hours, potential colonies of *S. uberis* were identified as being round, approximately one millimetre in diameter, and grey in colour. There was either an alpha (partial) or viridan (greenish colouration to the agar)

haemolysis, or gamma (no) haemolysis surrounding the colonies. Colonies with beta haemolysis (a clear ring of completely haemolysed cells surrounding the colony) were eliminated from consideration as being *S. uberis.*

After incubation at 37°C for 24 hours on Edwards agar, potential *S. uberis* colonies appeared as round dark brown colonies, less than 0.5mm in diameter (pin-point). The dark brown colour of the colonies was due in part to the darkness of the hydrolysed aesculin in the agar beneath the colony, which could be seen when the colony was removed from the agar surface. The agar itself lost its purple opaqueness and became a transparent gingery-brown colour.

2.3.2 INULIN BROTH

At the start of the study, the test for inulin fermentation was carried out in a liquid broth medium, as used by the Microbiology Laboratory of the Department of Veterinary Pathology, Glasgow University Veterinary School. This medium consisted of

5g 'Lab-Lemco' (Oxoid)
10g Bacteriological Peptone (Oxoid)
3g sodium chloride
2g disodium hydrogen phosphate
1l distilled water.

The pH was adjusted to 7.2 to 7.3, after which 12ml of bromothymol blue indicator (pH 6.0 yellow, pH 7.2 blue) was added. The indicator solution consisted of

0.1g bromothymol blue,
2.5ml 0.1N sodium hydroxide,
47.5ml distilled water.

After autoclaving the medium at 121°C for 15 minutes and cooling it to 52°C, ten grams of inulin were added. Three millilitre measures of the broth were then pipetted into Bijou bottles, each containing an inverted Durham tube. The filled bottles were then steamed for 20 minutes on each of three consecutive days (Tyndallisation).

Following incubation at 37°C for up to 48 hours in inulin broth, inulin fermenting organisms including *S. uberis* changed the colour of the broth from blue to yellow. When *S. uberis* was present in the broth medium, the colour change occurred within 24 hours. The inclusion of the Durham tubes in the Bijou bottles was quickly abandoned as the change in colour of the medium was sufficiently indicative of inulin fermentation.

2.3.3 INULIN SERUM AGAR

In the course of the study, the inulin broth medium was replaced by an inulin serum agar medium of Pugh and Bramley (1977), as described by Bramley *et al* (1979). This medium consisted of

34g BBL sugar free agar
10ml 0.5% bromocresol purple
(in 0.05N sodium hydroxide, pH 5.2 yellow, pH 6.8 purple)
2ml 0.1% crystal violet solution
1l distilled water.

After adjusting to a pH of 7.0 and autoclavation at 121°C for 15 minutes, the medium was cooled to 52°C, at which temperature five per cent sterile bovine serum (Gibco BRL, Life Technologies Ltd., P.O. Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF, Scotland) was added.

2.3.4 MODIFIED INULIN AGAR

Later in the study, the inulin medium of Pugh and Bramley (1977) was replaced by a modified inulin agar that did not include serum, but did include thallium acetate (Bramley, 1982). The medium consisted of

20g	Difco Bacto agar (Difco Laboratories, P.O. Box 1058, Detroit, Michigan 48232, USA)
2g	'Lab-Lemco' (Oxoid)
2.5g	Yeast Extract (Oxoid)
5g	Bacteriological Peptone (Oxoid)
10g	inulin
5g	sodium chloride
5g	potassium dihydrogen orthophosphate
0.75ml	Tween 80
0.05g	bromocresol purple
0.002g	crystal violet
1l	distilled water.

The medium was adjusted to a pH of 7.0 and, after autoclaving at 121°C for 15 minutes, one millilitre of 5.56% (w/v) thallium acetate solution was added per 100ml of molten agar (50°C).

2.3.5 MODIFIED INULIN AGAR WITHOUT THALLIUM ACETATE

The third inulin agar medium was exactly identical to the modified inulin agar of Bramley (1982) except that it did not contain thallium acetate.

On all three types of inulin agar, *S. uberis* grew as light grey or light purple colonies, approximately one millimetre in diameter. Inulin fermenting colonies other than *S. uberis* isolated from various farm milk samples, were coloured light grey, dark grey or purple; yellow colonies of *S. uberis*, as reported by Bramley (1982), were never seen. The presence of *S. uberis* caused most or all of the agar to turn yellow even when the colonies were sparsely scattered over the agar surface. Inulin fermenting colonies other than *S. uberis* produced a limited colour change in the agar, usually restricted to the immediate vicinity of the colony.

2.3.6 AGAR CONTAINERISATION AND STORAGE

All agars were hand poured into 90mm triple vent Petri dishes type 101VR20 (Sterilin Ltd., Lampton House, Lampton Road, Hounslow, Middlesex, TW3 4EE, England), each dish (also referred to as a plate) holding between 11 and 12ml agar. All plates were stored at 4°C until required.

2.3.7 SODIUM HIPPURATE MEDIA

The ability of bacterial isolates to hydrolyse sodium hippurate was examined by the method of Ayers and Rupp (1922) modified by Coffey and Foley (1937), using the ferric chloride test of Coffey and Foley (1937) as defined by Cullen (1967). The sodium hippurate medium of Ayers and Rupp (1922) modified by Coffey and Foley (1937) consisted of

1g	asparagine
5g	pepsin
0.03g	anhydrous calcium chloride
10g	sodium hippurate
1l	distilled water.

Three millilitre quantities of the medium were pipetted into Bijou bottles and autoclaved at 121°C for 15 minutes.

The ferric chloride test of Coffey and Foley (1937), as defined by Cullen (1967), was quantified by sequentially adding six 0.5ml quantities of acidified ferric chloride solution (0.7g of ferric chloride dissolved in 100ml of two per cent hydrochloric acid) to a control sample of three millilitres of uninoculated sodium hippurate media that had been incubated at 37°C for 24 hours. With the addition of the first 0.5ml ferric chloride a precipitate formed immediately; this progressively disappeared, but was not completely eliminated, as the remainder of the three millilitres of acidified ferric chloride solution was added. In sodium hippurate solutions inoculated with known *S. uberis* isolates and incubated for at 37°C for 24 hours, the precipitate remained even after six millilitres of acidified ferric chloride solution had been added.

During the study, a sodium hippurate agar medium, first used to differentiate *Klebsiella pneumoniae* and *Cloaca cloacae* (Thirst, 1957), was also tested. This medium was produced by adding ten grams New Zealand agar (undefined) and 2.5ml of four per cent (w/v) aqueous phenol red indicator (pH 6.8 yellow, pH 8.2 red) to the medium of Koser (1923). Koser's medium, used in sodium hippurate differentiation tests by Hajna and Damon (1934), consisted of

5g NaCl
0.2g $MgSO_4.7H_2O$
1g $(NH_4)H_2PO_4$
1g K_2HPO_4
3g sodium hippurate
1l distilled water.

After the pH of the media had been adjusted to between 6.8 to 7.0, it was autoclaved at 121°C for 15 minutes.

No bacterial growth on, or colour change of, the hippurate agar media (Thirst, 1957) was seen in up to seven days of incubation at 37°C following inoculation of the agar with confirmed *S. uberis* isolates. As the hippurate broth test was satisfactory, work to modify the hippurate agar test, so as to use it to differentiate hippurate hydrolysing from non-hippurate hydrolysing streptococcal colonies, was abandoned.

2.3.8 NUTRIENT BROTHS

Three nutrient broths, two of which were formulated as growing media for streptococci, were used during the study; Todd Hewitt broth (Oxoid), tryptose broth (Sharma and Packer, 1970), and a broth, developed during this study, consisting of equal volumes of Todd Hewitt broth and tryptose broth (THTP broth).

Todd Hewitt broth was prepared from Todd Hewitt medium (Oxoid) following the manufacturer's instructions. The formulation of Todd Hewitt medium consisted of

10g	'Lab-Lemco'
10g	peptone
1g	aesculin
5g	sodium chloride
0.0013g	crystal violet
0.33g	thallous sulphate
1l	distilled water.

After the pH of the broth had been adjusted to between 7.2 to 7.6, it was autoclaved at 121°C for 15 minutes.

The tryptose broth (Sharma and Packer, 1970) consisted of

3g 'Lab-Lemco' powder (Oxoid)

5g Yeast Extract (Oxoid)

10g Proteose-Peptone No 3 (Oxoid)

10g Tryptose (Oxoid)

5g sodium chloride

1l distilled water.

After the pH of the broth had been adjusted to 6.8, it autoclaved at 121°C for 15 minutes.

The THTP broth was produced by mixing equal volumes of both Todd Hewitt and tryptose broths before being autoclaved at 121°C for 15 minutes.

2.3.9 BROTH CONTAINERISATION AND STORAGE

Broths were either autoclaved in the glass containers in which the subsequent subculture took place (for example 30ml Universal containers) or in 500ml glass bottles. When cool, the lids were screwed down to make the containers air tight and, after cooling, the broth was stored at 4°C until required. Broth from a 500ml bottle distributed to smaller containers was re-autoclaved in the smaller containers.

2.3.10 INOCULATION OF AGAR PLATES

Inoculation of agar plates was carried out by a standard method (Gillies and Dodds, 1968): A microloop, sterilised in a Bunsen flame, was used to transfer a sample of liquid medium or a bacterial colony to a solid agar medium where it was spread over an small area near the circumference of the agar. The loop was flamed and drawn several

times over the inoculum on to an area of fresh agar adjacent to the initial inoculation area. This procedure was repeated two further times, each time using as inoculum the most distal part of the immediately preceding strokes, the loop being flamed before the inoculum was spread to a new area.

2.3.11 INOCULATION OF BROTH MEDIA

Measured quantities of milk were pipetted into known quantities of broth. Bacterial colonies were subcultured from agar plates by transferring a single, isolated colony from the plate to the broth (the broth container being held at an angle to prevent contamination dropping into the broth) by a microloop. The loop was then agitated in the broth and withdrawn. Swabs were likewise agitated in the broth before being discarded.

2.3.12 INOCULATION OF BOTH AGAR AND BROTH MEDIA

When a single colony from an agar medium was transferred to both another agar medium and to a broth medium, the colony was first spread over the initial small area of the agar medium by a microloop. The microloop was then agitated in the broth medium, flamed, and the pathogen spread over the remainder of the agar plate as detailed above.

2.3.13 CONFIRMATION OF IDENTITY OF ISOLATES

The identity of streptococcal isolates was confirmed by use of the API 20 STREP test (bio Mérieux, 1990). All isolates for the API tests were grown on blood agar and the tests were carried out according to the manufacturer's instructions.

2.3.14 STORAGE OF *STREPTOCOCCUS UBERIS* ISOLATES

Isolates of *S. uberis* were subcultured in Todd Hewitt broth at 37°C for 16 hours. One millilitre of the culture broth was transferred to an Eppendorf vial, after which one millilitre of 50% glycerol was added and the mixture gently shaken. The sealed Eppendorf vial was stored at -20°C (Hill and Leigh, 1989). To recover a viable sample, a hot loop was plunged into the frozen mixture, withdrawn and agitated in Todd Hewitt broth. The broth was then incubated at 37°C for 24 hours (Hill and Leigh, 1991).

2.4 INITIAL PROCEDURES ON MILK AND OTHER SAMPLES COLLECTED AT FARM VISITS

One of the aims of the study was to screen the milk samples for *S. uberis* using as few individual tests as possible, and hence various initial tests were investigated with each batch of milk samples. The initial screening processes included direct plating of samples on to blood agar, Edwards agar, or inulin agar followed by incubation at 37°C for up to 48 hours. Incubation of the milk samples or swabs in nutrient broth sometimes preceded the agar plating. The pattern of subsequent tests to isolate *S. uberis* varied in the course of the study and were dependent on the initial tests (appendix IV).

2.5 INVESTIGATIONS INTO THE CULTURAL CHARACTERISTICS OF KNOWN ISOLATES OF *STREPTOCOCCUS UBERIS*

Nine isolates of *S. uberis* were acquired from various sources; one as a freeze dried culture from the Microbiology Laboratory of the Department of Veterinary Pathology, Glasgow Veterinary School, two transported on milk agar slopes from the Scottish Agricultural Colleges' Veterinary Investigation Centre, Inverness, three transported on nutrient agar slopes from the Scottish Agricultural Colleges' Veterinary Investigation

Centre, Auchincruive, and three recovered from milk samples collected during the course of this study.

These isolates of *S. uberis* were subcultured on blood agar and, once having been confirmed as *S. uberis* by the API 20 STREP test, were used to test the reaction of the pathogen to all the media used in the study, to verify the cultural properties of the pathogen when growing on or in the various media, and to be the substrates for all other investigations.

2.6 *STAPHYLOCOCCUS AUREUS*

2.6.1 IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus cultures were identified by the size and colour of the colonies growing on blood agar after incubation at 37°C for up to 48 hours. Haemolytic and non-haemolytic strains were identified from the same cultures. Samples of suspected *Staph. aureus* colonies were placed in a drop of hydrogen peroxide on a glass slide. The immediate effervesence of oxygen confirmed the identification.

2.6.2 ANTIMICROBIAL SUSCEPTIBILITY TESTS FOR STAPHYLOCOCCI

When extensive growth of haemolytic and non-haemolytic staphylococci was found on incubated blood agar plates, individual colonies of all variants of morphology, colour and haemolysis were subcultured on two blood agar plates, the organism being spread across the entire surface of the agar (field plating) and two sets of seven antimicrobial susceptibility test discs (Oxoid) were placed on the agar by an Oxoid Dispenser Mark II before incubation at 37°C for 24 hours.

The 14 antimicrobial agents used (including standard abbreviations and concentrations) were

Ampicillin (AMP) 10μg

Chloramphenicol (C) 10μg

Lincomycin (MY) 2μg

Oxytetracycline (OT) 30μg

Penicillin G (P) 10 units

Streptomycin (S) 10μg

Sulphamethoxazole/Trimethoprim (Cotrimoxazole, SXT) 25μg

Amoxycillin (AML) 25μg

Amoxycillin/Clavulanic Acid 2:1 (AMC) 30μg

Cloxacillin (OB) 5μg

Cefuroxime Sodium (CXM) 5μg

Erythromycin (E) 10μg

Neomycin (N) 10μg

Novobiocin (NV) 5μg.

2.7 DNA TYPING OF *STREPTOCOCCUS UBERIS*

2.7.1 THE PROTOCOL OF HILL AND LEIGH (1989) AND SUBSEQUENT MODIFICATIONS

The DNA typing carried out in this study was based on the work of Hill and Leigh (1989). Following additional experimental work after publication of the paper on the DNA typing (fingerprinting) technique, the authors made several modifications to the protocol, which were made available at the start of this study (Hill and Leigh, 1991). Other modifications were made to the original technique because of unavailability of the

same equipment, or because it was found that a modification to the original protocol gave better results.

2.7.2 ISOLATION OF CHROMOSOMAL DNA

A culture of *S. uberis* was incubated at 37°C for 16 hours in ten millilitres of Todd Hewitt broth after which ten units of Hyaluronidase (Calbiochem Corporation, 10933 North Torrey Pines Road, La Jolla, California 92037, USA) per ml of broth were added. After reincubating the culture for a further 20 minutes, 1.5ml of the broth was transferred to an Eppendorf vial (in which all subsequent steps of the procedure were carried out), centrifuged at 5,000G for three minutes, resuspended in one millilitre of Tris (10mM) EDTA (5mM) buffer pH 7.8 and recentrifuged. After resuspension in 350μl of the same buffer, 200 units Mutanolysin (Sigma Chemical Company, 3050 Spruce Street, P.O. Box 14508, St. Louis, MO 63178, USA) was added. Following incubation for 30 minutes, the cells were lysed with the addition of 20μl SDS (20% SDS w/v in Tris [50mM] EDTA [20mM] pH 7.8) and three microlitres of Proteinase K (20mg/ml, Sigma) and incubated for one hour.

The protein was precipitated by the addition of 200μl saturated sodium chloride (approximately 6.0M) followed by gentle agitation for 15 seconds (Miller, Dykes and Polesky, 1988), and then removed by centrifugation (7,000G for ten minutes). The protein/salt pellet was discarded and the DNA precipitated by the addition of 2.5 volumes of cold 100% ethanol and 30μl sodium acetate.

The continuing poor separation of DNA and protein by the saturated sodium chloride method of Hill and Leigh (1989) led to it being discontinued towards the end of the study, and being replaced by the phenol chloroform extraction method, which the authors had originally used. After incubation with proteinase K, 350μl of phenol was

added to the contents of the Eppendorf vial and mixed for 30 minutes using a Blood Tube Rotator SB1 (Stuart Scientific Co. Ltd., Holmethorpe Avenue, Holmethorpe Industrial Estate, Redhill, Surrey, RH1 2NJ, England); the phenol layer was then aspirated off. After the phenol step had been repeated, 350μl of chloroform:isoamylalcohol (24:1) were added and mixed for 30 minutes as before. The chloroform:isoamylalcohol was then aspirated off and the step repeated, after which the DNA was precipitated from solution with ethanol and sodium acetate as in the original protocol.

The DNA precipitate was allowed to settle to the bottom, or stick to the side, of the Eppendorf vial, whereupon the supernatant was withdrawn using a Pasteur pipette. Any precipitate not at the bottom of the Eppendorf vial was transferred there by flash centrifugation, so that it was in prime position for contact with the rehydration buffer when the latter was added. The DNA precipitate was allowed to air dry before being rehydrated in 30μl Tris (10mM) EDTA (1mM) buffer pH 7.5 at 4°C for at least 24 hours (Hill and Leigh, 1989).

2.7.3 ENZYME DIGESTION OF DNA

The DNA was digested by *Hin*d III restriction endonuclease (Sigma). Five microlitres of the rehydrated DNA solution were placed in the bottom of an Eppendorf vial with three microlitres of distilled water, one microlitre of *Hin*d III restriction endonuclease (Sigma) and one microlitre of restriction endonuclease reaction buffer (Sigma), and incubated in a water bath at 37°C for eight hours. Following enzymic cleavage, two microlitres of electrophoresis marker (Sigma) was added to ten microlitres of the cleaved DNA/restriction endonuclease solution.

2.7.4 AGAROSE GEL ELECTROPHORESIS

The electrophoresis was carried out in Tris acetate running buffer (Tris acetate 0.04M, EDTA 0.001M) in wells formed in 20cm x 15cm gels containing one per cent low melting point agarose (Sigma). The wells were formed by positioning a 1.5mm 30 tooth comb (Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, England) in the tray before the molten gel was poured in, and leaving it there whilst the gel set. In order to be able to visualise the separated DNA by ultra violet light at the end of the electrophoresis, 20μl ethidium bromide solution (20mg/ml) was added to the molten agarose gel/Tris acetate buffer mixture just before it was poured into the electrophoresis cell.

The electrophoresis cell available for this study was a DNA Electrophoresis Cell (Bio-Rad Laboratories Ltd.). After the gel had been submerged in the running buffer, ten microlitres of the cleaved DNA/restriction endonuclease solution were pipetted into a well. The electrophoresis cell was initially run at 100 volts for 15 minutes to ensure the correct polarity of the electric field, as evidenced by the migration away from the wells of the electrophoresis marker, before being adjusted to 40 volts and left to run for 16 hours. Under the influence of the electric field cleaved segments of DNA migrate away form the wells through the gel at a speed proportional to their length, the longer segments of DNA moving slower than the shorter lengths. DNA segments of similar length migrate at the same speed and show up as distinct 'bands'.

In order to calibrate the length of the bands (in kilobases; Kb), *Hind* III digested lambda phage DNA molecular marker, consisting of four distinct lengths of DNA segments (4.4Kb, 6.5Kb, 9.4Kb and 23.0Kb), was placed in the outer wells of the gel.

At the end of the electrophoresis the bands of cleaved DNA in the gel were visualised by ultraviolet transillumination and photographed on polaroid film type 665.

2.8 OTHER INVESTIGATIONS

2.8.1 ASSESSMENT OF THREE NUTRIENT BROTHS FOR THE GROWTH OF *STREPTOCOCCUS UBERIS*

Three nutrient broths were compared to determine which would give the greatest harvest of viable *S. uberis* colony forming units. The nutrient broths used were Todd Hewitt broth (Oxoid), formulated as a growing medium for streptococci (Todd and Hewitt, 1932) and since used to culture isolates of streptococci for DNA typing (Hill and Leigh, 1989), and tryptose broth, used as a culture medium for the isolation of *S. uberis* from milk and skin swabs (Sharma and Packer, 1970). In addition, an equal volume of both Todd Hewitt and tryptose broths were mixed together to form a third broth called THTP broth. One colony of one of the known strains of *S. uberis* was subcultured on blood agar and, after incubation, single colonies were inoculated into ten millilitres of all three broths and incubated at 37°C for 24 hours. Equal volumes (approximately 3.3μl) were removed from the three broths by a microloop and spread on blood agar (Oxoid), Edwards agar (Oxoid) and inulin agar (Bramley, 1982), and all nine plates were incubated at 37°C for 24 hours. Colonies from all three blood agars that had been inoculated with the THTP broth were used as substrates for the API 20 STREP tests to check that no contamination had occurred. Colonies from the Edwards and inulin agars were similarly tested to see how the API 20 STREP test would perform with isolates grown on media other than blood agar.

2.8.2 ASSESSMENT OF THE NUMBER OF COLONY FORMING UNITS IN A COLONY OF *STREPTOCOCCUS UBERIS*

One colony of a known strain of *S. uberis* grown on blood agar was transferred to an Eppendorf vial containing one millilitre of sterile water and vortexed to obtain a uniform solution. From this solution 0.1ml was removed and mixed with 0.9ml of sterile water in another Eppendorf vial. Another four one in ten serial dilutions were carried out. From each of the dilutions, six drops of ten microlitres each, were placed separately on a blood agar plate, and all plates were incubated at 37°C for 24 hours. Following incubation, the number of colony forming units growing where each drop had been placed were counted visually, however the counting was only carried out where individual colonies were sufficiently separated to allow unaided visual counting.

CHAPTER THREE

RESULTS

3.1 THE FARMS VISITED

3.1.1 ACCESS TO FARMS

In the course of this study, a total of seven farms were visited with the intention of finding a commercial dairy herd with endemic *S. uberis* mastitis infection with which to carry out an epidemiological study of *S. uberis* using the DNA typing (fingerprinting) method of Hill and Leigh (1989). Two of the farms were already known to the Department of Veterinary Medicine, Glasgow University Veterinary School, and the other five farms were referred by veterinary practices. All the farmers were very willing to cooperate in the study as they were worried about the fact that if their milk had a high somatic cell count they would incur financial penalties imposed by the Scottish Milk Marketing Board. One farm, which happened to be the only farm of the seven that did not belong to the Scottish Milk Records Association (SMRA) Cell Count Advisory Service scheme, was referred by its veterinary practitioner because the Scottish Agricultural Colleges Veterinary Investigation Service had recently isolated *S. uberis* from milk samples collected from two cases of clinical mastitis on that farm.

During the study, Farm 1 was visited for sampling on five occasions, Farm 2 was visited twice, and Farms 3, 4, 5, 6 and 7 and were visited once each for sampling. The farms were visited in the following order, the first and second visits to Farm 1, both

visits to Farm 2, the third visit to Farm 1, the visits to Farms 3, 4, 5 and 6, the fourth and fifth visits to Farm 1, and finally the visit to Farm 7.

3.1.2 GENERAL DESCRIPTION OF THE FARMS VISITED

All seven farms visited were situated in the south-west of Scotland and they ranged in size from 45 to 180 hectares (appendix III). One farm was situated on the north west outskirts of Glasgow, whilst the other six were located in a quadrant lying to the east and south of Glasgow from the Midland Valley to Dumfriesshire. The dairy units were either the only enterprise on the farm or were incorporated into an integrated system usually involving sheep and/or cereals. The herd size ranged from 50 to 235 cows. Four of the herds (Farms 1, 2, 4 and 7) calved throughout the year, two herds (Farms 5 and 6) concentrated calving into the summer months (July to September) and the remaining herd (Farm 3) calved mainly in the spring.

With the exception of Farm 3, all the herds were milked in herringbone parlours and winter housed in cubicles with sawdust bedding, although on Farm 2, part of the herd was housed in straw yards. The Farm 3 herd was milked and winter housed in byres. All quarters of all cows were infused with dry cow intramammary therapy at the end of lactation.

3.1.3 CRITERIA FOR THE SELECTION OF COWS FOR SAMPLING

Six of the seven farms visited used the SMRA Cell Count Advisory Service. Farms 2, 5 and 6 were able to supply monthly SMRA records of the individual cow milk somatic cell counts for at least the previous six months. Farms 3 and 7 could only supply the most recent set of SMRA milk somatic cell counts. Although Farm 1 produced several months SMRA records, only one related to a recent milk sampling, the others all

detailing cell counts of milk samples collected at least 11 months previously. For the first three visits to Farm 1, and the visits to Farms 2 and 3, the available records were used to select cows with a recent history of high milk somatic cell counts, and quarter milk samples were collected from those cows. Farm 4 did not participate in the SMRA scheme and quarter milk samples were collected from the half of the herd that entered on one side of the milking parlour. On the remaining visits, a composite udder milk sample was drawn from the milking machine collecting jar when each cow had finished milking.

The owners of Farms 3, 6 and 7 reported that the incidence of clinical mastitis was very low in their herds and, in the cases of Farms 6 and 7, this was consistent with a low herd somatic cell count. Although examination of the SMRA records of the herds on Farms 6 and 7 showed that it was unlikely that they would be affected with *S. uberis* mastitis, a sampling visit was still arranged not only to try to find undetected subclinical *S. uberis* mastitis but also in order not to refuse to accept a referral from a veterinary practice.

3.2 LABORATORY TECHNIQUES FOR THE ISOLATION AND IDENTIFICATION OF *STREPTOCOCCUS UBERIS*

3.2.1 DIFFERENTIAL MEDIA USED TO ISOLATE AND IDENTIFY *STREPTOCOCCUS UBERIS*

Streptococcus uberis was isolated and identified by its cultural properties when grown on various agar and in various broth media; blood agar (Oxoid), Edwards agar (Oxoid), inulin broth (Microbiology Laboratory, Department of Pathology, Glasgow University Veterinary School), inulin serum agar (Pugh and Bramley, 1977; Bramley *et al*, 1979), inulin agar (Bramley, 1982) and sodium hippurate broth (Ayers and Rupp, 1922; Coffey

and Foley, 1937; Cullen, 1967) (appendix IV). Todd Hewitt broth (Oxoid), tryptose broth (Sharma and Packer, 1970) and THTP broth, a mixture of equal volumes of Todd Hewitt and tryptose broths developed during this study, were used as enrichment media. API 20 STREP tests (bio Mérieux, 1990) were used to confirm the identity of isolates of *S. uberis* and to identify other organisms.

3.2.2 BLOOD AGAR

Colonies of known isolates of *S. uberis* growing on blood agar were grey in colour, less than one millimetre in diameter and were often surrounded by a zone of greenish discolouration of the agar (viridan haemolyis), but never by alpha or beta haemolysis (figure 1).

3.2.3 EDWARDS AGAR

During the early part of the study it became apparent that colonies growing on Edwards agar that were of any appreciable size (over 0.5mm diameter), and which caused darkening of the agar beneath or beneath and around the colony due to hydrolysis of the aesculin, were not *S. uberis*. Incubation of known isolates of *S. uberis* on Edwards agar at 37°C for up to 48 hours produced very small "pin-point" colonies which, far from darkening the adjacent aesculin agar, caused the whole plate to loose its opaque purple colour and to become a translucent "washed out" pale ginger colour (figure 2). Larger colonies, causing the normally described appearance of aesculin hydrolysis, were usually identified as *Aerococcus viridans* (*A. viridans*).

A

B

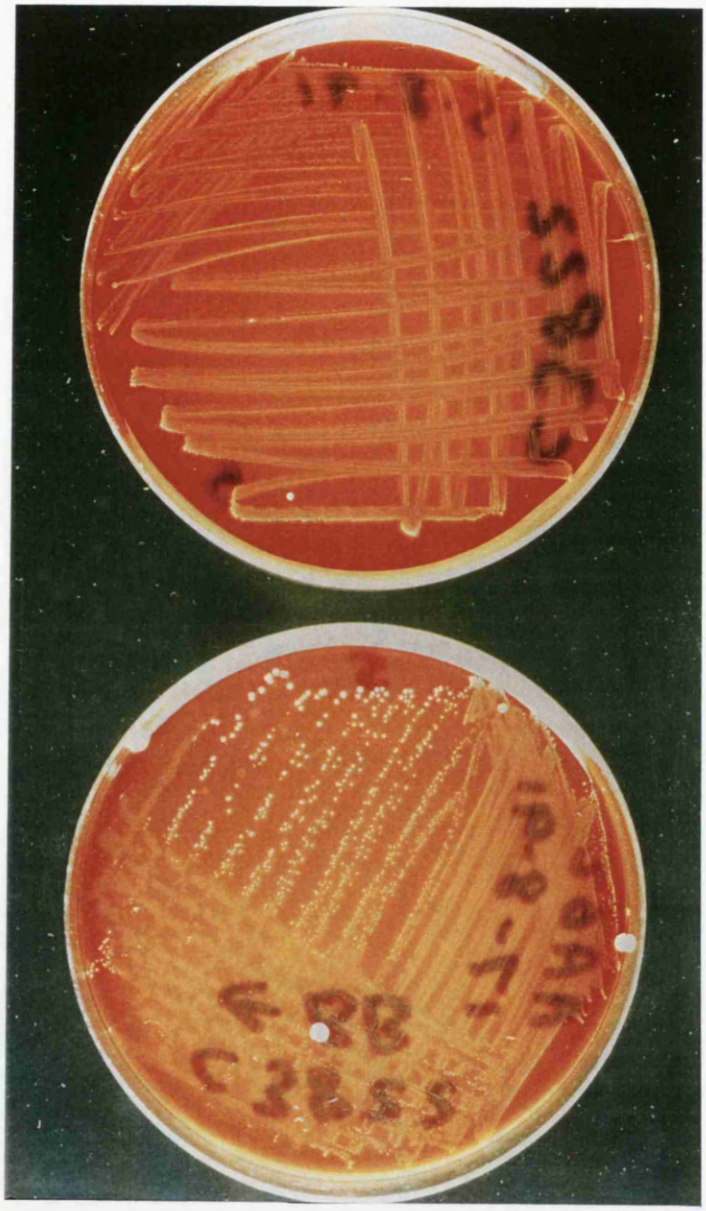


Figure 1 *S. uberis* incubated on blood agar

A - *S. uberis* subcultured directly on blood agar

B - *S. uberis* incubated in THTP broth before being subcultured on blood agar

A

B

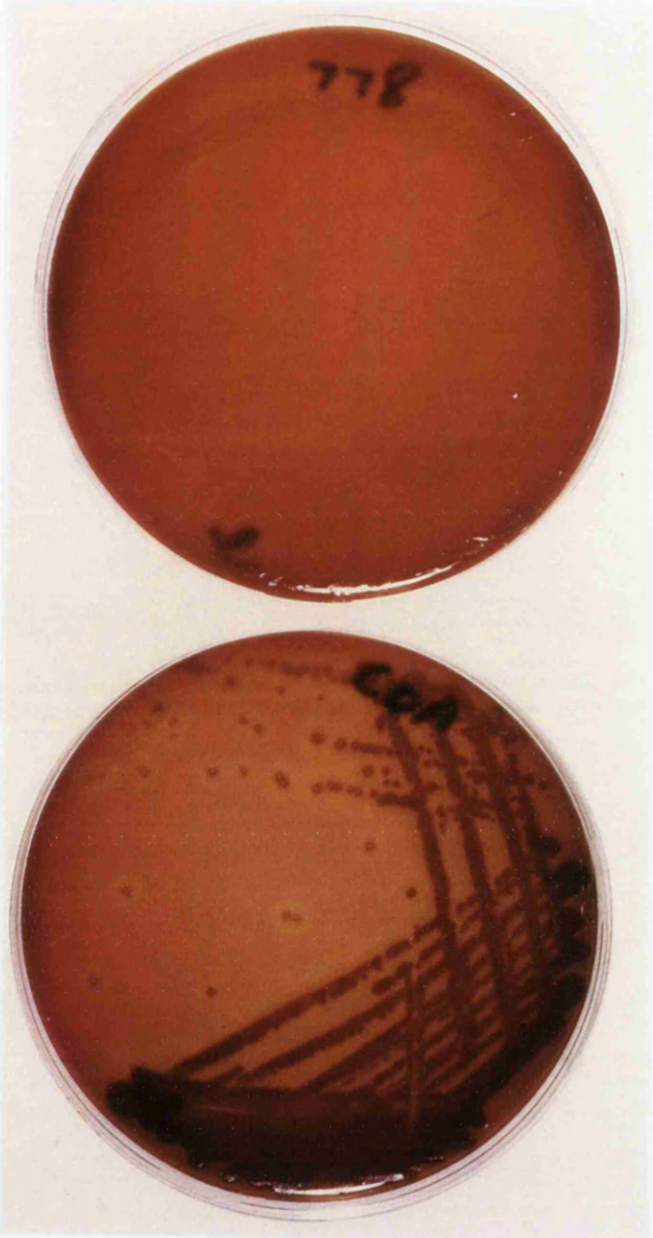


Figure 2 Aesculin hydrolysis test

A - pin-point sized colonies of *S. uberis* (visible in lower left quadrant) growing on Edwards agar with no blackening of the media

B - colonies of *A. viridans* growing on Edwards agar with characteristic blackening of the media

3.2.4 INULIN MEDIA

For the samples collected from the farm visits up to and including Farm 4, the inulin fermentation test was carried out using inulin broth (Microbiology Laboratory, Department of Pathology, Glasgow University Veterinary School). Bromothymol blue, the pH indicator included in the broth, changed colour from blue to yellow in response to a reduction of the pH caused by fermentation of the substrate by inulin metabolising isolates (figure 3). In an experiment carried out during the isolation procedures on milk samples from Farm 5 using identical subcultures incubated in inulin broth and on inulin serum agar (Pugh and Bramley, 1977), it was found that, whilst many isolates fermented the inulin in both media as seen by the change of colour of the bromothymol blue indicator in the broth and the bromocresol purple indicator in the agar, a significant number of isolates fermented the inulin in the agar without fermenting it in the broth, and no isolate fermented inulin in the broth without also fermenting it in the agar. Similarly, during the isolation procedures on milk samples from Farm 6, more isolates caused the bromocresol purple indicator in the inulin agar (Bramley, 1982) to change colour than in the inulin serum agar. Inulin agar continued to be the inulin medium used in the isolation procedures carried out on the milk samples collected from the fourth and fifth visits to Farm 1 and from Farm 7 (figure 4). Identical subcultures of isolates recovered from the milk samples collected from Farm 7 were incubated on inulin agar (Bramley, 1982) and a modified version containing no thallium acetate. No difference was seen in the growth or fermentation abilities of the isolates.

3.2.5 SODIUM HIPPURATE BROTH

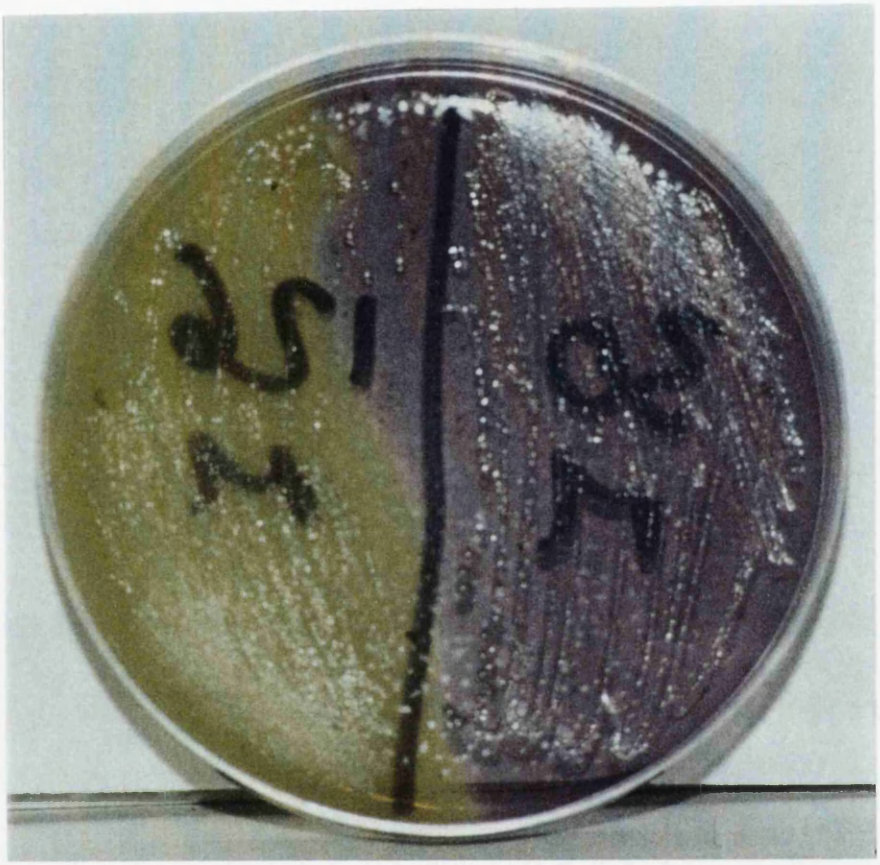
The ability of bacterial isolates to hydrolyse sodium hippurate was examined by the method of Ayers and Rupp (1922) modified by Coffey and Foley (1937), using the ferric chloride test of Coffey and Foley (1937) as defined by Cullen (1967). After the

1 2

Figure 3 *S. uberis* inulin fermentation test in broth medium containing bromothymol blue indicator

1 - before incubation

2 - after incubation



1 2

Figure 4 Inulin fermentation test using inulin agar medium containing bromocresol purple indicator

1 - inulin fermenting *S. uberis*

2 - non inulin fermenting bacteria

bacterial isolate had been incubated in three millilitres of sodium hippurate broth at 37°C for 24 hours, three millilitres of ferric chloride solution was added in 0.5ml volumes. If no hydrolysis of the hippurate had occurred, the initial precipitate formed by the addition of the first 0.5ml of ferric chloride would almost disappear with the addition of the remaining 2.5ml, leaving only the precipitate of the protein constituant of the broth covering the bottom of the Bijou bottle (figure 5). If the isolate had hydrolysed the hippurate to benzoate, the precipitate formed by the addition of 0.5ml ferric chloride would persist after the addition of the remaining 2.5ml ferric chloride and would quickly settle to the bottom of the Bijou bottle as a distinct flocculent mass.

3.2.6 CHANGES IN THE ISOLATION PROTOCOL

In the course of the study, the various broth and agar media were changed in order to obtain the most efficient procedure for selectively isolating and identifying *S. uberis* (appendix IV). The initial isolation procedure, carried out on the samples collected during the first three visits to Farm 1 and the visits to Farms 2, 3 and 4, was to incubate the samples on blood agar and/or Edwards agar. For samples collected from Farms 5 and 6, the initial procedure was to incubate 0.5ml of milk in five millilitres of Todd Hewitt Broth. The broth cultures from samples collected from Farm 5 were subcultured on Edwards agar. As it was found that many aesculin hydrolysing species of bacteria thrived on Edwards agar to the possible detriment of the pin-point size colonies of *S. uberis*, the first differential test carried out on milk samples collected during the visit to Farm 6, the last two visits to Farm 1 and the visit to Farm 7, was changed from hydrolysis of aesculin to fermentation of inulin.

Following the visit to Farm 6 and before the fourth visit to Farm 1, it was found that incubating a known isolate of *S. uberis* in THTP broth gave a greater harvest of *S. uberis* colony forming units than when identical isolates were incubated in Todd Hewitt broth

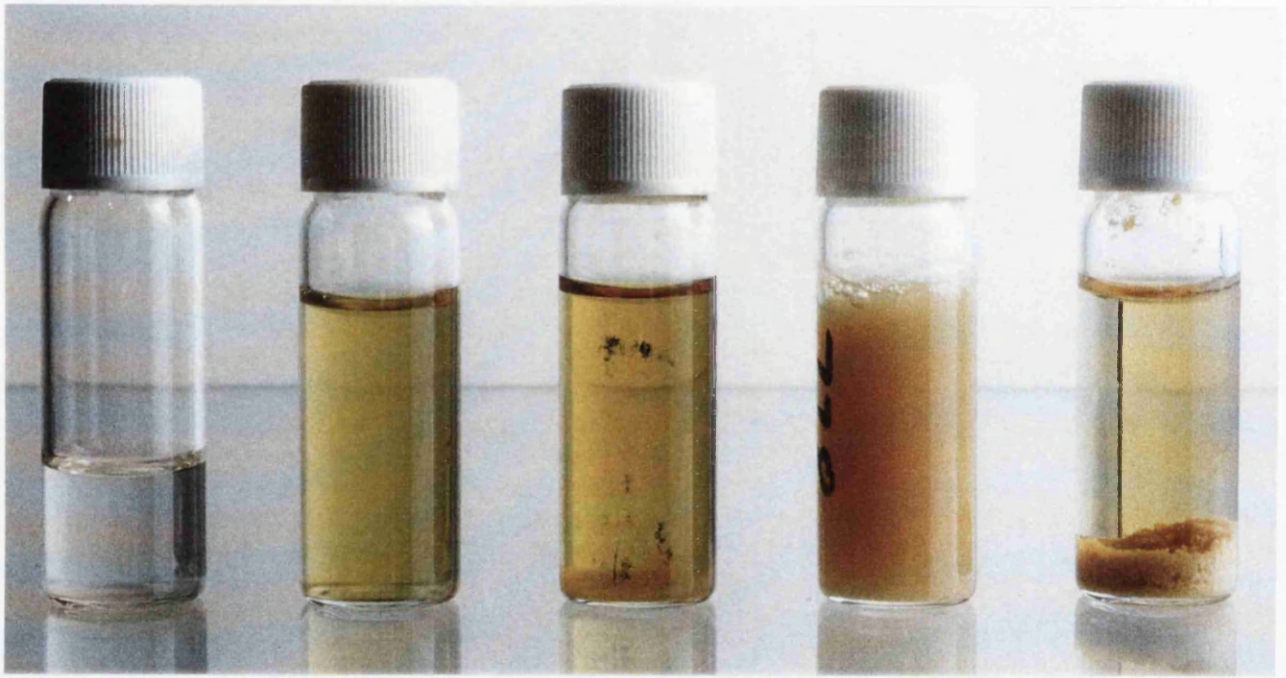


Figure 5 Sodium hippurate test

1 - 3ml sodium hippurate broth

2 - 3ml ferric chloride solution added to 3ml incubated uninoculated sodium hippurate broth

3 - result when non hippurate hydrolysing isolate incubated in the broth, protein in the broth being precipitated

4 & 5 - result when hippurate hydrolysing *S. uberis* incubated, producing benzoate precipitate which settles on standing

or tryptose broth (3.11.1 Assessment of Three Nutrient Broths for the Growth of *Streptococcus uberis*). THTP broth replaced Todd Hewitt broth in the initial incubation of milk samples collected from the fourth and fifth visits to Farm 1 and from Farm 7.

In addition, using the milk samples collected during the fourth visit to Farm 1, it was found that following incubation of 0.5ml of milk in both five and ten millilitres of THTP broth, and subculture of identical quantities of the milk broth mixtures on inulin agar, a greater number of inulin fermenting colonies grew on the agars subcultured with milk incubated in ten millilitres of THTP broth than from milk incubated in five millilitres of the broth (see Farm 1, visit 4). The 0.5ml milk to ten millilitres THTP broth ratio was used as the initial broth incubation procedure on the milk samples collected from the fifth visit to Farm 1, and from Farm 7.

3.3 FARM 1

3.3.1 FARM AND HERD MANAGEMENT

This farm was situated to the north-west of Glasgow. In addition to a 90 Friesian cow dairy herd, sheep, pig, horse and deer enterprises were maintained on 100 hectares (appendix III). Calving occurred throughout the year although most of the cows calved in the autumn. The cows were individually identified by freeze brand number, but many of the numbers were illegible and the new entrants to the herd remained unbranded.

The dairy herd was winter housed in cubicles, that were too short for the Friesian cows using them, with no head rail or lunging space at the head end. Many cows sought more comfortable lying space in the passageways and feeding area. The herd was milked in a herringbone parlour. Before milking, teats and udders were cleaned by

hosing with a dilute solution of "Iosan Superdip" (0.5% w/w available iodine and ten per cent Glycerine B.P., 75ml Superdip diluted in four litres water; Ciba-Geigy Agrochemicals, Whittlesford, Cambridge, CB2 4QT, England) and dried with individual paper towels. After milking, the teats were sprayed with undiluted "Iosan Superdip". Cows were not made to stand for any length of time after milking. Routine farm treatment of mild mastitis cases consisted of two intramammary infusions of 300mg (300,000 units) procaine penicillin G ("Mylipen Q.R. '300'"; Pitman-Moore Ltd., Crewe Hall, Crewe, Cheshire, CW1 1YR, England) with an interval of 48 hours between infusions. Dry cow therapy consisted of 300mg (300,000 units) procaine penicillin G in an oily base ("Mylipen Dry Cow"; Pitman-Moore Ltd.) infused into every quarter at the end of each cow's lactation.

3.3.2 FARM 1 VISIT 1 18.03.1991

3.3.3 SELECTION OF COWS FOR SAMPLING

Only the most recent SMRA individual cow milk somatic cell count, measured three weeks previously, was used to select the cows to be sampled, all other available cell count records being over 11 months old (appendix V). At the most recent SMRA cell count, 50 cows had been in milk and the average herd milk somatic cell count was 246,000 cells per ml (appendix VI). Fourteen cows (28% of the herd) had a milk somatic cell count of over 250,000 cells per ml of which eight cows (16%) had a cell count of over 600,000 cells per ml.

From the 14 cows recorded as having a milk somatic cell count of over 250,000 cells per ml at the most recent SMRA visit, individual quarter milk samples were collected from the first nine cows that entered the milking parlour. Of these, two cows had milk somatic cell counts of between 250,000 and 400,000 cells per ml and seven cows had

cell counts of over 600,000 cells per ml. One cow with a cell count of less than 100,000 cells per ml was also sampled to see if a cow showing no clinical signs of *S. uberis* mastitis and a lower than herd average milk somatic cell count could be harbouring the pathogen. Vaginal and rectal swabs were collected from the same ten cows to see if *S. uberis* could be isolated from these orifices and to assess the procedural difficulties of sampling quarters, vaginas and rectums at the same visit.

3.3.4 BACTERIAL ISOLATION AND IDENTIFICATION

Thirty-six milk samples, nine vaginal swabs and nine rectal swabs were collected from the nine cows sampled. All milk samples and swab material were incubated on Edwards agar at 37°C for up to 48 hours; aesculin hydrolysing colonies were subcultured on blood agar, and also inoculated into five millilitres of Todd Hewitt broth (appendix VII). Following incubation of the agars and broths at 37°C for 24 hours, the broths in which no growth had occurred, together with the corresponding blood agars, were discarded. From the remaining blood agars, single colonies showing alpha, gamma or viridan haemolysis were inoculated into inulin broth and incubated at 37°C for up to 48 hours.

Source	Milk	Vagina	Rectum
Total number of samples	36	9	9
Isolates that hydrolysed aesculin	28	8	10
Aesculin hydrolysing isolates that grew in Todd Hewitt broth	18	2	5
Non-beta haemolytic isolates that hydrolysed aesculin, grew in Todd Hewitt broth and fermented inulin	15	1	3

Table 3.1 Analysis of samples collected from Farm 1 visit 1

The 19 inulin fermenting isolates were identified using the API 20 Strep test as either *A. viridans* or *Listeria* species or could not be identified.

Source	Number of Isolates	Bacterial Identification
Milk isolates	11	*A. viridans*
	4	*A. viridans* / *Listeria* species
Vaginal isolates	1	no identification
Rectal isolates	1	*A. viridans*
	2	*A. viridans* / *Listeria* species

Table 3.2 Analysis of inulin fermenting isolates recovered from samples collected at Farm 1 visit 1

Colonies that produced a wide variety of patterns of haemolytic rings were also found growing on the Edwards agar. They usually fermented inulin but were identified by the API 20 STREP test as *A. viridans* or *Listeria* species, or could not be identified.

Having noted the similarity of growth on blood agar and Edwards agar of the samples, further tests on the samples from two cows (cows 87 and 104) were not carried out. All milk samples were also incubated on blood agar at 37°C for 24 hours. On this occasion, minimal growth of *Staph. aureus* occurred. A milk somatic cell count of over one million cells per ml recorded for one cow (Cow 47) indicated possible recent udder infection; the complete lack of any organism growth from the milk samples from this cow may have been due to antibacterial treatment in the recent past, although this was not mentioned at the visit.

3.3.5 FARM 1 VISIT 2 22.04.1991

3.3.6 SELECTION OF COWS FOR SAMPLING

Before the second sampling visit took place, the two most recent monthly SMRA individual cow milk somatic cell count records were available for analysis. The latest SMRA visit recorded 54 cows as being in milk, and the average milk somatic cell count had risen to 328,000 cells per ml (appendix V; appendix VI). Twenty-one cows (39% of the herd) had a milk somatic cell count of over 250,000 cells per ml. Six of the eight cows with a cell count of over 600,000 cells per ml at the last recording continued to be in that band, which had increased to 12 cows (22%) of which nine (16.5%) had cell counts in excess of one million cells per ml.

From the 21 cows recorded as having a milk somatic cell count of over 250,000 cells per ml at the most recent SMRA visit, individual quarter milk samples were collected from the first 15 cows that entered the milking parlour.

3.3.7 BACTERIAL ISOLATION AND IDENTIFICATION

Fifty-nine quarter milk samples, collected from 15 cows (one cow had only three functioning teats), were incubated on blood agar at 37°C for up to 48 hours; only 11 milk samples, from eight cows, produced colonies of streptococcal appearance (appendix VIII). These were subcultured on Edwards agar and incubated at 37°C for up to 48 hours. Five of the eleven Edwards plates showed no growth, two had colony growth with no aesculin hydrolysis, and the remaining four had colony growth with aesculin hydrolysis; however the latter colonies were over two millimetres in diameter with a variety of haemolytic rings surrounding them and subsequent API 20 STREP tests showed them to be *A. viridans.*

Samples/Isolates	Number
Milk samples collected	59
Milk samples from which streptococcal-like organisms grew on blood agar	11
Isolates subcultured on Edwards agar that grew with no aesculin hydrolysis	2
Isolates subcultured on Edwards agar that grew with aesculin hydrolysis	4
Aesculin hydrolysing isolates identified by API 20 Strep test as *A. viridans*	4

Table 3.3 Analysis of milk samples collected from Farm 1 visit 2

The most prevalent pathogens found growing on the blood agar were haemolytic and non-haemolytic staphylococci of varying colony size and colour, 39 quarters from 14 of the 15 cows being infected. Five representative examples of the *Staph. aureus* colonies were subcultured on blood agar for antimicrobial susceptibility tests (Oxoid). On this occasion only seven antimicrobial agents were included (in all other susceptibility tests in this study, 14 antimicrobial agents were used). The pathogens were found to be more resistant to Penicillin G, Ampicillin and Streptomycin than to the other four antibacterial agents tested namely Chloramphenicol, Lincomycin, Oxytetracycline and Sulphamethoxazole/Trimethoprim (Co-Trimoxazol) (appendix IX). The complete lack of any organism growth from the milk from one cow (cow 18) may have been due to treatment in the recent past, although this was not mentioned at the visit.

3.3.8 FARM 1 VISIT 3 13.05.1991

3.3.9 SELECTION OF COWS FOR SAMPLING

The third consecutive monthly SMRA individual cow milk somatic cell count record showed that the 46 cows then in milk had an average cell count of 303,000 cells per ml (appendix V; appendix VI). Fifteen cows (32.5% of the herd) had a milk somatic cell count of over 250,000 cells per ml. Seven of the twelve cows with a cell count of over 600,000 cells per ml at the previous recording continued to be in that band; three of these had recorded a cell count of over 600,000 cell per ml for the last three months. Three cows in the same band had been dried off and a further five cows registered a cell count of over 600,000 cells per ml to keep the total number of cows in that band at 12 (26%).

This sampling was undertaken to ascertain whether the milk somatic cell count reflected the presence of staphylococcal organisms in the milk samples. From the 15 cows recorded as having a milk somatic cell count of over 250,000 cells per ml at the most recent SMRA visit, individual quarter milk samples were collected from the first nine cows that entered the milking parlour. One cow (cow 89), with a milk somatic cell count of less than 250,000 cells per ml and with no clinical signs of *Staph. aureus* mastitis, was also sampled from all four quarters to see whether her udder was harbouring the pathogen.

3.3.10 BACTERIAL ISOLATION AND IDENTIFICATION

Forty quarter milk samples, collected from ten cows, were incubated on blood agar at 37°C for up to 48 hours. Twenty-two of the agar plates showed a significant growth of

Staph. aureus, identified by size and morphology of the colonies, and the effervesence produced when the colonies were mixed with hydrogen peroxide (catalase test).

Cow	Milk Somatic Cell Count (cells per ml)	Quarters infected with *Staph. aureus*
10	1,256,000	3
14	616,000	2
16	1,435,000	4
31	606,000	4
34	916,000	2
39	602,000	2
47	3,082,000	1
72	162,000	0
82	447,000	2
99	267,000	3

Table 3.4 Prevalence of *Staph. aureus* infection in milk samples collected from ten cows at Farm 1 visit 3

The one milk sample with a somatic cell count of less than 250,000 cells per ml (cow 72) was the only one from which no *Staph. aureus* could be isolated, and the cow with the highest cell count (cow 47) had only one quarter from which *Staph. aureus* could be isolated.

3.3.11 FARM 1 VISIT 4 29.08.1991

3.3.12 SELECTION OF COWS FOR SAMPLING

Due to a change in farm management policy, the monthly SMRA individual cow milk somatic cell count recording had been discontinued after the April 1991 recording, so no recent milk somatic cell count records were available for scrutiny. Composite udder milk samples, from all 67 cows in milk, were drawn from the milking machine collecting jars.

3.3.13 BACTERIAL ISOLATION AND IDENTIFICATION

Composite udder milk samples were drawn from the milk collecting jars for all 67 cows in milk. Following the experiment that showed that *S. uberis* grew better in THTP broth (a mixture of equal volumes of Todd Hewitt broth and tryptose broth) than in Todd Hewitt broth or tryptose broth alone (3.11.1 Assessment of Three Nutrient Broths for the Growth of *Streptococcus uberis*), 0.5ml of each milk sample was incubated in both five and ten millilitres of THTP broth at 37°C for 40 hours, before being subcultured on inulin agar (Bramley, 1982) and incubated at 37°C for up to 48 hours (appendix X).

Source	Number
Milk samples	67
Milk samples from which inulin fermenting organisms grew after incubation:-	
in 10ml THTP broth only	17
in 5ml THTP broth only	4
in both volumes of broth	17

Table 3.5 Comparison of the recovery of inulin fermenting colonies using two different volumes of THTP broth from milk samples collected at Farm 1 visit 4

Inulin fermenting colonies were not recovered from 29 of the 67 milk samples, irrespective of the volume of THTP broth used. The assessment of growth of inulin fermenting organisms was made by visually comparing the amount of colour change, from purple to yellow, of the inulin agar surrounding the colonies. From 17 of the milk samples, inulin fermenting colonies were recovered from incubation in ten millilitres of THTP broth but not from incubation in five millilitres of broth, and from another four milk samples the position was reversed with inulin fermenting colonies being recovered after incubation in five millilitres THTP broth but not from incubation in ten millilitres of broth. Of the 17 milk samples from which inulin fermenting colonies were recovered from incubation in both five and ten millilitres THTP broth, eight milk samples produced a greater number of inulin fermenting colonies from ten millilitres of THTP broth than from five millilitres of broth, and six milk samples produced a greater number of inulin fermenting colonies from five millilitres of THTP broth than from ten millilitres of broth; three milk samples produced comparably equal numbers of inulin fermenting colonies from both volumes of broth. In all, 55 plates produced inulin fermenting

colonies. Many non-inulin fermenting organisms also grew on the agar and some of these deepened the purple colour of the agar.

Specimen inulin fermenting colonies from all 55 inulin plates were inoculated into THTP broth and incubated at 37°C for 24 hours, and those isolates that retained their viability were then subcultured on both Edwards agar and blood agar. After incubation at 37°C for up to 48 hours, isolates that grew on the blood agar as small grey colonies surrounded by alpha, gamma or viridan haemolysis and grew as pin point colonies on Edwards agar, which became a translucent pale ginger colour, were subcultured from the blood agar into sodium hippurate broth and incubated at 37°C for 24 hours.

Isolates	Number
Inulin fermenting isolates	55
Potential *S. uberis* isolates growing on blood agar	42
Potential *S. uberis* isolates growing on Edwards agar	20
Potential *S. uberis* isolates growing on both blood and Edwards agar	4
Isolates that hydrolysed sodium hippurate	3

Table 3.6 Analysis of the inulin fermenting isolates recovered from milk samples collected at Farm 1 visit 4

Both isolates (from Cows 30 and 45) positive to the inulin, aesculin and hippurate tests and that showed the required morphology and haemolysis on blood agar, were confirmed as *S. uberis* by the API 20 STREP test. The same test failed to identify another inulin, aesculin and hippurate positive isolate that produced large mucoid colonies on blood agar. Two hippurate negative isolates, also tested by the API 20 STREP test, were identified as *S. bovis* and *Enterococcus avium.*

3.3.14 FARM 1 VISIT 5 11.09.1991

3.3.15 SELECTION OF COWS FOR SAMPLING

This sampling took place two weeks after the previous sampling. All 70 cows in milk were sampled by means of a composite udder milk sample drawn from the collecting jar. In addition, quarter milk samples were collected from the two cows (cows 30 and 45) from whose composite udder milk samples, collected at the previous visit, *S. uberis* had been isolated.

3.3.16 BACTERIAL ISOLATION AND IDENTIFICATION

Composite udder milk samples were drawn from the milk collecting jars for all 70 cows in lactation. Individual quarter samples were collected from cows 30 and 45, from whose composite milk samples *S. uberis* had been isolated on the previous sampling visit. From the isolation procedures carried out on the previous set of milk samples from this herd, it had been found that 25 of the 67 milk samples produced inulin fermenting colonies either exclusively, or in greater quantity, when 0.5ml milk was incubated in ten millilitres of THTP broth at 37°C for 40 hours, whereas only ten milk samples responded in the same way from incubation in five millilitres of broth. Therefore 0.5ml quantities of the milk samples were incubated only in ten millilitres of THTP broth before being subcultured on inulin agar (Bramley, 1982) and incubated at 37°C for up to 48 hours (appendix XI).

Inulin fermenting colonies were inoculated into THTP broth at 37°C for 24 hours and then subcultured on Edwards agar and incubated at 37°C for up to 48 hours. Potential *S. uberis* isolates growing on the Edwards agar were subcultured on blood agar and incubated at 37°C for up to 48 hours. Non beta haemolytic isolates growing on the blood agar were incubated in sodium hippurate broth at 37°C for up to 48 hours.

Samples/Isolates	Number
Milk samples containing inulin fermenting organisms	27
Inulin fermenting isolates that hydrolysed aesculin	15
Aesculin hydrolysing isolates that grew on blood agar as colonies of < 1mm diameter without beta haemolysis	1
Number of blood agar isolates that hydrolysed sodium hippurate	1

Table 3.7 Analysis of the milk samples collected from Farm 1 visit 5

The one potential *S. uberis* isolate hydrolysed sodium hippurate and was confirmed by the API 20 STREP test as being *S. uberis* (isolate 35A/O). *Streptococcus uberis* was not recovered from any of the jar or quarter milk samples collected from the two cows (cows 30 and 45) from which *S. uberis* had been previously isolated. As far as was known, no treatment had been given to the two cows in the intervening time.

3.4 FARM 2

3.4.1 FARM AND HERD MANAGEMENT

Farm 2, of about 200 hectares, was situated in Lanarkshire, and was already known to the Department of Veterinary Medicine, Glasgow University Veterinary School. The main enterprise was a herd of 235 Jersey cows, each individually named as well as carrying a numbered neck collar. The herd was theoretically divided into three subherds according to milk yield. The high yielding cows in early lactation, and the low yielding cows in late lactation were winter housed in cubicles that were too long for Jersey cows and hence the sparse sawdust bedding was often soiled. Cows in mid lactation were

winter housed in straw yards. The relative sizes of the subherds was determined by the housing available and not by the number of cows in the high, middle or low yield bands.

Calving occurred throughout the year and, due to extended dry periods between the end of one lactation and the next calving, the calving index (the time between two successive calvings) for many cows was greater than one year. Clinical mastitis was reported to be a frequent occurrence, although numbers of cases were not recorded, and cows with a persistent infection were put to suckling calves or had their lactation prematurely terminated.

The herd was milked in a herringbone parlour. No pre-milking teat and udder washing was carried out unless the teat ends were grossly dirty, when only hosing with water was used to clean them; this had the incidental disadvantage that the milker's hands could remain visibly dirty as occurred throughout the part of the afternoon milking that was observed during the preliminary visit. After milking, the teats were sprayed with "Deosan Super Ex-Cel" (0.5% w/w available iodine, with glycerine and sorbitol as emollients; Deosan Ltd., Weston Favell Centre, Northampton, NN3 4PD, England). Routine farm treatment of mild mastitis cases consisted of three intramammary infusions of one gram (1,000,000iu) procaine penicillin G and 0.5g dihydrostreptomycin sulphate ("Streptopen Milking Cow"; Pitman-Moore Ltd.) at 12 hour intervals. Dry cow therapy consisted of one gram (1,000,000iu) procaine penicillin G and 0.5g dihydrostreptomycin sulphate in a long acting oily base ("Streptomycin Dry Cow"; Pitman-Moore Ltd.) infused into every quarter at the end of each cow's lactation.

3.4.2 FARM 2 VISIT 1 15.04.1991

3.4.3 SELECTION OF COWS FOR SAMPLING

Before the sampling visit, the previous seven consecutive monthly SMRA individual cow milk somatic cell count records were made available for analysis (appendix XII). The owners reported that they were having to resort to heavy culling of the persistently high somatic cell count cows to keep the average cell count below the level at which a penalty would be imposed on their milk returns. Analysis of all seven SMRA cell count records showed that many cows had a persistently high cell count (over 600,000 cells per ml) for several consecutive months and, despite dry cow therapy, some cows that ended their lactation with a high cell count continued to have a similar cell count at the start of their next lactation. The most recent SMRA records showed that 145 cows had been recorded with an average milk somatic cell count of 453,000 cells per ml; this had risen from 315,000 cells per ml in the last three months. Sixty-seven cows (46% of the herd) had a milk somatic cell count of over 250,000 cells per ml of which 35 cows (24%) had a cell count of over 600,000 cells per ml.

Seventy-nine quarter samples were collected from 20 cows (one cow had only three functioning teats), 16 of the cows having had a cell count of over 600,000 cells per ml at one of the last two SMRA cell count recordings. Two other cows selected had cell counts between 250,000 and 400,000 cells per ml and the remaining two cows, with a history of chronic mastitis, were sampled at the farmer's request. Care was taken to ensure that cows were selected from all three yield groups and thus from both housing systems (cubicles with sawdust bedding, and yards with straw bedding).

3.4.4 BACTERIAL ISOLATION AND IDENTIFICATION

Seventy-nine quarter milk samples were collected from 20 cows, one cow having only three functioning teats. All milk samples were incubated on blood agar at 37°C for up to 48 hours (appendix XIII).

Samples/Isolates	Number
Quarter milk samples	79
Milk samples from which *Staph. aureus* was isolated on blood agar	66
Milk samples from which streptococcal-like organisms grew on blood agar	55
Streptococcal-like isolates that hydrolysed aesculin	44
Aesculin hydrolysing isolates with colonies of < 1mm diameter	4

Table 3.8 Analysis of milk samples collected from Farm 2 visit 1

Sixty-six milk samples produced a wide variety of haemolytic and non-haemolytic, coagulase positive staphylococci on blood agar, the colonies being coloured white, cream or yellow. Fifty-five milk samples grew colonies on blood agar that initially appeared to be streptococci but many of them grew to over one millimetre in diameter within 24 hours of incubation at 37°C and many had a distinct wide band of haemolysis surrounding them. Non beta haemolytic colonies of less than one millimetre diameter were subcultured on Edwards agar at 37°C for up to 48 hours and the four isolates that grew as aesculin hydrolysing colonies of less than one millimetre in diameter were subcultured on blood agar for identification by the API 20 STREP test; some isolates could not be identified but those that were identified were found to be *A. viridans*. API 20 STREP tests carried out on larger aesculin hydrolysing colonies showed that they were also *A. viridans*.

3.4.5 FARM 2 VISIT 2 01.05.1991

3.4.6 SELECTION OF COWS FOR SAMPLING

From analysis of the results of the first visit, it was realised that subclinical staphylococcal mastitis was playing a significant role in causing the elevation of the milk somatic cell counts on this farm. As no antimicrobial sensitivity tests had been carried out on the staphylococcal strains isolated from the milk samples from the first visit, a second visit was arranged, two weeks after the first visit, to collect milk samples specifically to isolate *Staph. aureus* and obtain an antimicrobial sensitivity profile. Fifteen milk samples were collected from the quarters of the first eight cows coming into the parlour that had been sampled on the first visit and been found to be infected with *Staph. aureus*. In addition, at the owners request, a cow that had recently suffered from clinical mastitis (Cow 117) was also sampled, from all four quarters.

3.4.7 BACTERIAL ISOLATION AND IDENTIFICATION

Nineteen quarter milk samples collected from nine cows were incubated on blood agar at 37°C for 24 hours. Four quarters showed no bacterial growth on blood agar, possibly because the quarters had been treated with antibacterial medication in the time between the two visits. Thirteen examples of staphylococcal colonies, chosen on their variety of colour and type of haemolysis, were selected from the blood agar plates, and antimicrobial susceptibility tests (Oxoid) carried out (appendix XIV). The milk samples from all four quarters of Cow 117 produced *Staph. aureus* growth. Examples of colonies from the two most severely clinically affected quarters were included in the antimicrobial tests. Ten isolates were resistant to penicillin G and ampicillin of which seven were also resistant to amoxycillin. The only other isolate that was resistant to

amoxycillin was one of the three isolates sensitive to penicillin G and ampicillin. All 13 isolates were sensitive to the amoxycillin/ clavulanic acid combination.

3.5 FARM 3 VISIT 20.05.1991

3.5.1 FARM AND HERD MANAGEMENT

This farm of about 40 hectares with other non-adjacent rented land, was situated close to the M8 motorway linking Glasgow and Edinburgh. Beside the herd of 50 Friesian cows, the farm also ran a flock of sheep. Most of the herd calved in the spring. All the cows were identified by freeze brand number and were winter housed in byres, the standings being bedded with straw. The farmer reported that only an occasional case of clinical mastitis was seen.

The cows were milked at their standings in the byres by bucket milking machine. Pre-milking preparation consisted of washing the teats and udders with a dilute solution of "Deosan Super Iodip" (2% w/w available iodine, with glycerol and sorbitol as emollients, 3.5ml diluted in one litre of water; Deosan Ltd.) using a common udder cloth, the teats being dried with the wrung-out cloth. After milking, the teats were dipped in undiluted "Deosan Super Iodip". Routine farm treatment of mild mastitis cases consisted of three intramammary infusions of "Streptopen Milking Cow" (Pitman-Moore Ltd.) at 12 hour intervals or three daily infusions of a combination of 150mg penethamate hydrochloride, 150mg dihydrostreptomycin, 50mg framycetin sulphate and 5mg prednisolone ("Leo Yellow Milking Cow"; Leo Laboratories Ltd., Longwick Road, Princes Risborough, Aylesbury, Buckinghamshire, HP17 9RR, England). Dry cow therapy consisted of "Streptopen Dry Cow" (Pitman-Moore Ltd.) infused into every quarter at the end of each cow's lactation.

3.5.2 SELECTION OF COWS FOR SAMPLING

The first SMRA visit had taken place less than two weeks before the sampling visit, and the cows to be sampled were chosen on the basis of this one set of milk somatic cell count records. The 32 cows tested at the SMRA visit had an average somatic cell count of 426,000 cells per ml based on 31 milk samples, one sample being sour (appendix XV). Thirteen cows (41% of the herd) had a milk somatic cell count of over 250,000 cells per ml. Eight cows (25%) had a cell count of over 600,000 cells per ml and all eight were the oldest cows in the herd (between seven and nine years of age). Individual quarter milk samples were collected from ten of the 12 cows that had recorded a cell count of over 250,000 cells per ml and that were still in milk.

3.5.3 BACTERIAL ISOLATION AND IDENTIFICATION

Thirty-five quarter milk samples were collected from ten cows, three cows had three functioning quarters and one cow only two functioning quarters; all cows sampled were between seven and nine years of age. All milk samples were incubated on blood agar and Edwards agar at 37°C for up to 48 hours (appendix XVI).

Samples/Isolates	Number
Milk samples producing colonies on Edwards agar	15
Milk samples producing aesculin hydrolysing colonies on Edwards agar	4
Aesculin hydrolysing isolates that fermented inulin (broth)	0

Table 3.9 Analysis of milk samples collected from Farm 3

Most of the colonies of streptococcal appearance that grew on the Edwards agar were translucent when held to the light (no dark area of hydrolysed aesculin under the colony) and were much bigger in diameter than the pin-point size of *S. uberis* colonies after 24 hours incubation, and more than one millimetre in diameter after 48 hours incubation. The four isolates that grew as aesculin hydrolysing colonies were isolated into inulin broth and incubated at 37°C for up to 48 hours, but none showed any ability to ferment the substrate.

Haemolytic and non-haemolytic staphylococcal colonies appeared on 31 of the 35 blood agar plates. Twelve staphylococcal colonies, chosen for their variation of colour and haemolytic properties, were tested for antimicrobial susceptibility (Oxoid). Three strains were resistant to penicillin G, two to ampicillin and amoxycillin, and one to cloxacillin, but all were sensitive the amoxycillin/clavulanic acid combination (appendix XVII).

3.6 FARM 4 VISIT 01.07.1991

3.6.1 FARM AND HERD MANAGEMENT

This south Ayrshire farm of about 80 hectares maintained a herd of 120 Friesian cows calving throughout the year, and winter housed in cubicles bedded with sawdust. The herd was not in the SMRA Cell Count Advisory Service scheme, and the Scottish Agricultural Colleges Veterinary Investigation Centre had recently isolated *S. uberis* from milk samples collected from two cases of clinical mastitis. All the cows were identified by numbered ear tags.

The herd was milked in a herringbone parlour. Before milking the teats and udders were hand washed with a hose delivered dilute solution of "Deosan Super Iodip" and the teats

were dried with individual paper towels. After milking the teats were sprayed with "Deosan Super Iodip". Routine farm treatment of mild mastitis cases consisted of three intramammary infusions of 50mg of clavulanic acid, 200mg of amoxycillin and ten milligrams of prednisolone ("Synulox Lactating Cow Intramammary Suspension"; Smithkline Beecham Animal Health Ltd., now Pfizer Ltd.) at 12 hour intervals. Dry cow therapy consisted of 100mg of framycetin sulphate, 100mg of penethamate hydriodide and 300mg of procaine penicillin ("Leo Red Dry Cow"; Leo Laboratories Ltd.) infused into every quarter at the end of each lactation.

3.6.2 SELECTION OF COWS FOR SAMPLING

As the herd did not have individual cow milk somatic cell counts monitored by the SMRA, there were no counts to which to refer in order to select cows to be sampled. Eighty-one cows were in milk at the time of the visit including two cows (cows 71 and 126) that, in the recent past, had suffered from clinical mastitis from which *S. uberis* had been isolated. Individual quarter samples were collected from these two cows together with the half of the herd that were milked on one side of the herringbone parlour. In total, 169 quarter milk samples were collected from 43 cows, three cows each having only three functioning teats. The quarter milk somatic cell counts were carried out by the Haematology Laboratory, Department of Pathology, Glasgow University Veterinary School, using a Coulter Counter model ZF (Coulter Electronics), from which the average somatic cell count for the milk from each cow was calculated. The average quarter milk somatic cell count from the 43 cows that were sampled was 593,000 cells per ml (appendix XVIII). Twenty-seven cows (63% of the cows tested) had a milk somatic cell count of over 250,000 cells per ml, of which 12 (28%) had a cell count of over 600,000 cells per ml. The two cows from whose milk *S. uberis* had been isolated had udder average cell counts of 186,000 (cow 71) and 202,000 (cow 126) cells per ml, and none of the quarter cell counts from these two cows was above 297,000 cells

per ml. High udder cell counts were, with one exception, due to one or two quarters having a very high cell count; the one exceptional udder had cell counts in all four quarters ranging from one million to three million cells per ml.

3.6.3 BACTERIAL ISOLATION AND IDENTIFICATION

The 169 quarter milk samples, collected from 43 cows, were incubated on blood agar at 37°C for up to 48 hours.

Samples/Isolates	Number
Milk samples collected	169
Milk samples from which *Staph. aureus* were recovered (blood agar)	94
Milk samples from which streptococci were recovered	59
Streptococccal isolates that hydrolysed aesculin (Edwards agar)	5

Table 3.10 Analysis of milk samples collected from Farm 4

Fifty-nine of the milk samples grew colonies of streptococcal appearance on the blood agar (appendix XVIII). When these were subcultured on Edwards agar and incubated at 37°C for 24 hours, only five isolates grew colonies that hydrolysed aesculin. After being subcultured on blood agar and incubated at 37°C for up to 48 hours, all five isolates were identified by the API 20 STREP test as being *L. lactis*. The lack of growth of any organisms from all four quarter samples from cow 71 (one of the cows that had a history of recent clinical *S. uberis* mastitis) was assumed to be due to antibacterial treatment still being effective within the udder.

Ninety-four milk samples produced either haemolytic or non-haemolytic staphylococci after incubation on blood agar. Antimicrobial susceptibility tests (Oxoid) on 16 of these isolates, chosen for their range of colour and haemolytic properties, showed that three were resistant to penicillin, and two were resistant to ampicillin (appendix XIX). All were sensitive to the amoxycillin/clavulanic acid combination. However, one isolate was resistant to nine of the fourteen antibiotics, including amoxycillin, cloxacillin and erythromycin, and only moderately sensitive to the amoxycillin/clavulanic acid combination.

3.7 FARM 5 VISIT 16.07.1991

3.7.1 FARM AND HERD MANAGEMENT

This farm of over 140 hectares in south-west Lanarkshire maintained a herd of 120 Friesian cows and, in addition, had sheep and arable enterprises. The cows were winter housed in cubicles with sawdust bedding, and milked in a herringbone parlour. Most of the herd calved in the period from July to September. All cows were identified by freeze brand number.

Pre-milking teat cleaning consisted of wiping the teats with a proprietary udder towel impregnated with polyhexamethylene bisguanide hydrochloride (3.3%) and alkyl dimethyl benzyl ammonium chloride (7.7%) ("Medicated Teat Towels"; Genus Animal Health, Cleeve House, Lower Wick, Worcester, WR2 4NS, England) that was claimed to be effective for use on up to 25 cows. No disinfectant solution was applied to the teats after milking. Clinical mastitis had been common in this herd, so much so that various antibacterial treatments under veterinary supervision had been abandoned in favour of homeopathic medication added to the drinking water. The farmer could not remember the original ingredients of the medication. Dry cow therapy at the end of lactation had

been continued and consisted of "Leo Red Dry Cow" or 500mg cloxacillin ("Orbenin Dry Cow Intramammary Suspension"; Smithkline Beecham).

3.7.2 SELECTION OF COWS FOR SAMPLING

Before the sampling visit, the previous eight consecutive monthly SMRA individual cow milk somatic cell count reports were available for analysis (appendix XX). At the most recent SMRA visit, the 83 cows sampled had an average milk somatic cell count of 523,000 cells per ml. The previous eight herd average cell counts had ranged between 449,000 and 760,000 cells per ml. Forty-seven cows (57% of the herd) had milk somatic cell counts of over 250,000 cells per ml, of which 26 cows (31%) had a cell count of over 600,000 cells per ml. Thirty-five cows had registered cell counts of over 600,000 cells per ml for at least two of the last three recordings, and ten of these cows had counts of over 600,000 cells per ml in all of the last four recordings. A similar picture was observed over the whole eight months of the cell count recordings.

At the sampling visit, 101 cows were in milk and a composite udder milk sample was drawn from the collecting jar after each cow had been milked. In addition, udder skin swabs were collected from all cows, the side of the udder facing the parlour pit being sampled.

3.7.3 BACTERIAL ISOLATION AND IDENTIFICATION

Composite udder milk samples were drawn from the milk collecting jar after each of the one hundred and one cows had been milked. Swabs from the udder skin were also collected from the same cows.

All 101 milk samples were incubated on blood agar at 37°C for up to 48 hours; in addition, 0.5ml of each sample was incubated at 37°C for 16 hours in five millilitres Todd Hewitt broth before being subcultured on Edwards agar and incubated at 37°C for up to 48 hours (appendix XXI).

The skin swabs were gently vortexed in their transport broth (Todd Hewitt broth) before being removed, and the broths were incubated at 37°C for 16 hours after which they were subcultured on Edwards agar and incubated at 37°C for up to 48 hours.

Samples/Isolates	Number (Milk)	Number (Skin)
Samples	101	101
Aesculin hydrolysing isolates (Edwards agar)	32	38
Aesculin hydrolysing isolates that fermented inulin (broth & agar)	12	12
Aesculin hydrolysing isolates that fermented inulin (agar only)	6	13
Inulin fermenting isolates that hydrolysed sodium hippurate	3	2

Table 3.11 Analysis of milk and skin samples collected from Farm 5

Thirty-two milk samples and 38 skin swabs produced aesculin hydrolysing colonies of less than one millimetre diameter on Edwards agar. These were subcultured into Todd Hewitt broth and incubated at 37°C for 12 hours, then inoculated into inulin broth and subcultured on inulin serum agar (Bramley *et al*, 1979), and both sets of inulin media were incubated at 37°C for up to 48 hours. Twelve isolates from the milk samples and 12 isolates from the skin swabs fermented the inulin in both broth and agar media. An additional six isolates from the milk samples and 13 isolates from the skin swabs fermented the inulin in the agar but not in the broth. No isolate fermented inulin in the

broth without also fermenting it in the agar. As the agar medium produced more inulin fermenting isolates and, in addition, had the advantage of allowing individual inulin fermenting colonies to be subcultured, no further tests were carried out using the inulin broth. Inulin fermenting colonies from the inulin serum agar were inoculated into Todd Hewitt broth and incubated at 37°C for 12 hours before being inoculated into sodium hippurate broth and incubated at 37°C for up to 48 hours. No isolate hydrolysed hippurate.

Of the 37 cows from which inulin fermenting organisms were isolated, only six cows had inulin fermenting organisms isolated from both milk and skin. Twelve cows had inulin fermenting organisms in the milk only and 19 cows had inulin fermenting organisms on the udder skin only.

Staphylococcus aureus was found to be the predominant organism growing on all blood agar plates inoculated with milk. Antibacterial susceptibility tests (Oxoid) on 15 isolates chosen for their variety of colour and haemolytic properties showed them all to be very sensitive to penicillin G, ampicillin and amoxycillin (appendix XXII).

3.8 FARM 6 VISIT 30.07.1991

3.8.1 FARM AND HERD MANAGEMENT

This mixed enterprise farm of 70 hectares, with another 20 hectares of rented land, lay between Glasgow and Edinburgh several miles to the south of the M8 motorway. Beside the herd of 60 Friesian cows the farm supported a bull beef enterprise and a flock of sheep; some cereals were also grown. The cows were winter housed in cubicles with sawdust bedding. Most cows calved in the months of July and August. All cows were

identified by freeze brand number. The farmer reported the prevalence of clinical mastitis to be very low.

The cows were milked in a herringbone parlour. Pre-milking teat and udder preparation was carried out using a hose delivered dilute solution of "Iosan Teat Dip" (1.55% w/w available iodine, 15% w/w Glycerine B.P., 25ml "Iosan Teat Dip" diluted in four litres water; Ciba-Geigy Agrochemicals), and after milking the teats were dipped in "Iosan Teat Dip" (one part "Iosan Teat Dip" diluted in two parts water). Routine farm treatment of mild mastitis cases consisted of three intramammary infusions of "Streptopen Milking Cow" at 12 hour intervals, and dry cow therapy consisted of "Streptopen Dry Cow" infused into every quarter at the end of each cow's lactation.

3.8.2 SELECTION OF COWS FOR SAMPLING

Before the sampling visit, the previous nine consecutive monthly SMRA individual cow milk somatic cell count reports were available for analysis (appendix XXIII). At the most recent SMRA visit, the 30 cows sampled gave a herd average of 238,000 cells per ml of which ten cows (33%) had a milk somatic cell count of over 250,000 cells per ml, but only one of these (three per cent) had a milk somatic cell count of over 600,000 cells per ml; no cow had a cell count of over one million cells per ml. The previous seven herd average cell counts, when there had been between 51 and 67 cows in milk, had ranged between 165,000 and 236,000 cells per ml. The one bulk milk cell count previous to that, when most cows had been dried off and only 18 cows were in milk, was 554,000 cells per ml. Composite udder milk samples from all 39 cows in milk were drawn from the milking machine collecting jars.

3.8.3 BACTERIAL ISOLATION AND IDENTIFICATION

Composite udder milk samples were collected from the milk collecting jars for each of the 39 cows in milk. All milk samples were incubated on blood agar at 37°C for up to 48 hours; in addition, 0.5ml of each milk sample was incubated in five millilitres Todd Hewitt broth at 37°C for 16 hours before being subcultured on to both inulin serum agar (Bramley *et al*, 1979) and inulin agar (Bramley, 1982) and incubated at 37°C for up to 48 hours (appendix XXIV).

Milk Samples	Inulin Serum Agar	Inulin Agar
Number incubated	39	39
Number producing colonies	35	38
Number producing inulin fermenting colonies	4	6

Table 3.12 Comparison of results using inulin serum agar (Bramley *et al*, 1979) and inulin agar (Bramley, 1982)

Both inulin fermenting colonies and colonies that did not ferment inulin grew on both inulin serum agar (Bramley *et al*, 1979) and inulin agar (Bramley, 1982), although inulin fermenting isolates originating from the same milk sample grew only on one or other of the agars and never on both. With one exception, the inulin fermenting colonies on the inulin serum agar grew on only the primary streaked area of the agar and the medium changed colour from blue to yellow only as a narrow ring around each colony; the one exception changed the medium colour over the first three streaked areas. In contrast, the inulin fermenting colonies on the inulin agar, once again with one exception, appeared on all four sets of streakings and hence changed the colour of the indicator to yellow throughout most of the agar medium, the exception only producing a colour

change in the area of the first streaking. However, when the inulin fermenting colonies from both types of inulin agar were subcultured on Edwards agar and incubated at 37°C for 24 hours none of the colonies showed aesculin hydrolysing properties and were too large (greater than one millimetre in diameter) to be *S. uberis*. This was subsequently confirmed by the API 20 STREP test, the isolates being either *S. faecalis*, *Enterococcus avium* or could not be identified.

Although staphylococcal colonies were present on most of the blood agar plates they appeared only as isolated colonies. No antimicrobial susceptibility tests were carried out.

3.9 FARM 7 VISIT 29.10.1991

3.9.1 FARM AND HERD MANAGEMENT

This large lowland farm of over 160 hectares in Dumfriesshire maintained a herd of 120 Friesian cows, the other main enterprise being cereal growing. The cows were winter housed in cubicles with sawdust bedding. Calving occurred throughout the year. All cows were identified by freeze brand number. The prevalence of clinical mastitis was reported to be very low.

The cows were milked in a herringbone parlour. Before milking, teats and udders were cleaned by hosing with a dilute solution of "Deosan Super Iodip" and dried with individual paper towels. After milking, the teats were dipped in undiluted "Deosan Super Iodip". Routine farm treatment of mild mastitis cases consisted of three intramammary infusions of "Streptopen Milking Cow" at 12 hour intervals, and dry cow therapy consisted of "Streptopen Dry Cow" infused into every quarter at the end of each cow's lactation.

3.9.2 SELECTION OF COWS FOR SAMPLING

Only the most recent SMRA individual cow milk somatic cell count, recorded one month previously, was available for examination. The herd average milk somatic cell count from the 114 cows then in lactation was 289,000 cells per ml (appendix XXV). Forty-nine cows (43% of the herd) had a milk somatic cell count of over 250,000 cells per ml, and 18 cows (16%) had a cell count of over 600,000 cells per ml, with six cows (five per cent) being over one million cells per ml. Composite udder milk samples from the 114 cows in lactation were taken from the milking machine collecting jars.

3.9.3 BACTERIAL ISOLATION AND IDENTIFICATION

Composite udder milk samples were drawn from the milk collecting jars for all 114 cows in milk. All 114 milk samples were incubated on inulin agar (Bramley 1982) at 37°C for up to 48 hours; in addition, 0.5ml of each milk sample was incubated in ten millilitres of THTP broth at 37°C for 16 hours, and then subcultured on inulin agar (Bramley, 1982) both with, and without, the incorporation of thallium acetate, and incubated at 37°C for up to 48 hours (appendix XXVI). Laboratory procedures were directed solely towards isolating streptococci.

Samples/Isolates	Number
Milk samples collected	114
Isolates able to ferment inulin (agar) without preliminary THTP broth stage	0
Isolates able to ferment inulin (agar) after preliminary THTP broth stage:-	
thallium acetate included in agar	34
thallium acetate not included in agar	34
Inulin fermenting isolates that grew on blood agar	19
Non beta haemolytic colonies < 1mm diameter	5
Non beta haemolytic isolates that hydrolysed aesculin	0

Table 3.13 Analysis of milk samples collected from Farm 7

No inulin fermenting colonies grew on inulin agar that had been directly inoculated with the milk samples. Of the milk samples previously incubated in THTP broth, 34 produced inulin fermenting colonies. Using visual examination, the presence or absence of thallium acetate made no difference to the appearance or growth of inulin fermenting colonies or any other colonies. Inulin fermenting colonies were subcultured on blood agar and incubated at 37°C for 24 hours. The five isolates that grew as small non-beta haemolytic colonies were subcultured on Edwards agar and incubated at 37°C for 24 hours. Three of these isolates failed to grow on the Edwards agar and colonies of the other two isolates failed to hydrolyse aesculin. Contaminating organisms overgrew several of the blood agar plates rendering them useless.

3.10 DNA TYPING OF *STREPTOCOCCUS UBERIS*

3.10.1 ISOLATION OF CHROMOSOMAL DNA

In this study, the various strains of *S. uberis* were typed by a method based on the DNA restriction endonuclease (fingerprint) method of Hill and Leigh (1989). After the bacterial cells had been lysed, the addition of saturated sodium chloride was supposed to have extracted and precipitated the nucleic proteins, leaving the DNA to be precipitated from solution by ethanol and sodium acetate. The DNA was then digested by restriction endonuclease and the fragments separated into bands by gel electrophoresis. The pattern of these bands formed the 'fingerprint'.

The method of Hill and Leigh (1989), using saturated sodium chloride to precipitate out the DNA proteins, was found to give very unsatisfactory results, with only infrequent recovery of DNA, most of which still had protein attached that nullified any digestion by the restriction endonuclease, and resulted in a 'smear' effect when the electrophoresis of the DNA was visualised (figure 6). Following extraction of the protein with phenol and chloroform/isoamyl alcohol, as originally used by Hill and Leigh (1991), DNA was almost invariably recovered and in greater quantity, although this latter distinction was only assessed visually.

3.10.2 AGAROSE GEL ELECTROPHORESIS OF DNA

Agarose gel electrophoresis separated the cleaved lengths of DNA which, when visualised, produced an unique pattern of bands or 'fingerprints' for each different strain of *S. uberis*. The lengths of the DNA in the bands was calibrated by the *Hin*d III digested lambda phage DNA molecular marker, consisting of four distinct lengths of

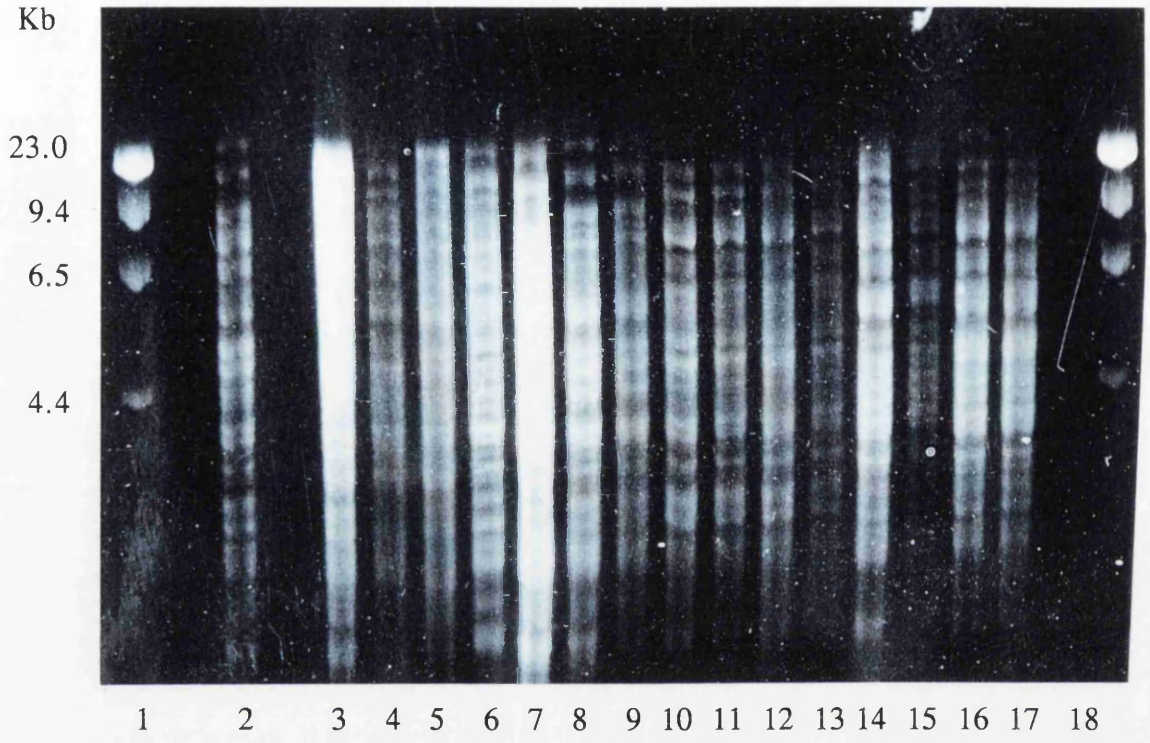


Figure 6 Differentiation of *S. uberis* isolates by DNA typing

Wells are at the top with DNA fractions migrating downwards, the smaller fractions migrating further than the larger fractions

Tracks 3, 5, and 7 'smeared' due to protein attached to the DNA

Pattern of tracks 8 and 9 differ from tracks 10 to 17

Tracks 1 and 18 contain *Hin*d III digested lambda phage DNA molecular marker to calibrate the fraction bands in kilobases (Kb)

DNA segments (4.4Kb, 6.5Kb, 9.4Kb and 23.0Kb), that had been placed in the outer wells of the gel and that moved through the gel under the influence of the electric field.

The differences in the band patterns were mainly restricted to the larger DNA fragments (6.5 to 23.0 kilobases), the bands of fragments of less than 9.4 kilobases were homologous amongst the strains (figure 6).

3.11 OTHER INVESTIGATIONS

3.11.1 ASSESSMENT OF THREE NUTRIENT BROTHS FOR THE GROWTH OF *STREPTOCOCCUS UBERIS*

Three nutrient broths, Todd Hewitt, tryptose and THTP (a mixture of equal volumes of Todd Hewitt and tryptose broths) were compared to determine which would give the greatest harvest of viable *S. uberis* colony forming units. Single colonies of a known strain of *S. uberis*, grown on blood agar, were inoculated into equal volumes of all three broths and incubated at 37°C for 24 hours. After shaking to obtain even distribution of bacteria throughout the broths, equal volumes of the three broths were then subcultured on blood agar, Edwards agar and the inulin agar of Bramley (1982), and incubated at 37°C for 24 hours.

After incubation it could be clearly seen by unaided visual examination that inoculum from the THTP broth had produced the greatest colony growth on all three agars; growth was better from the inoculum incubated in the tryptose broth than that incubated in the Todd Hewitt broth. Colony growth from all three broths was best on the blood agar plates, and there was better colony growth on the inulin agar than on the Edwards agar (figure 7).

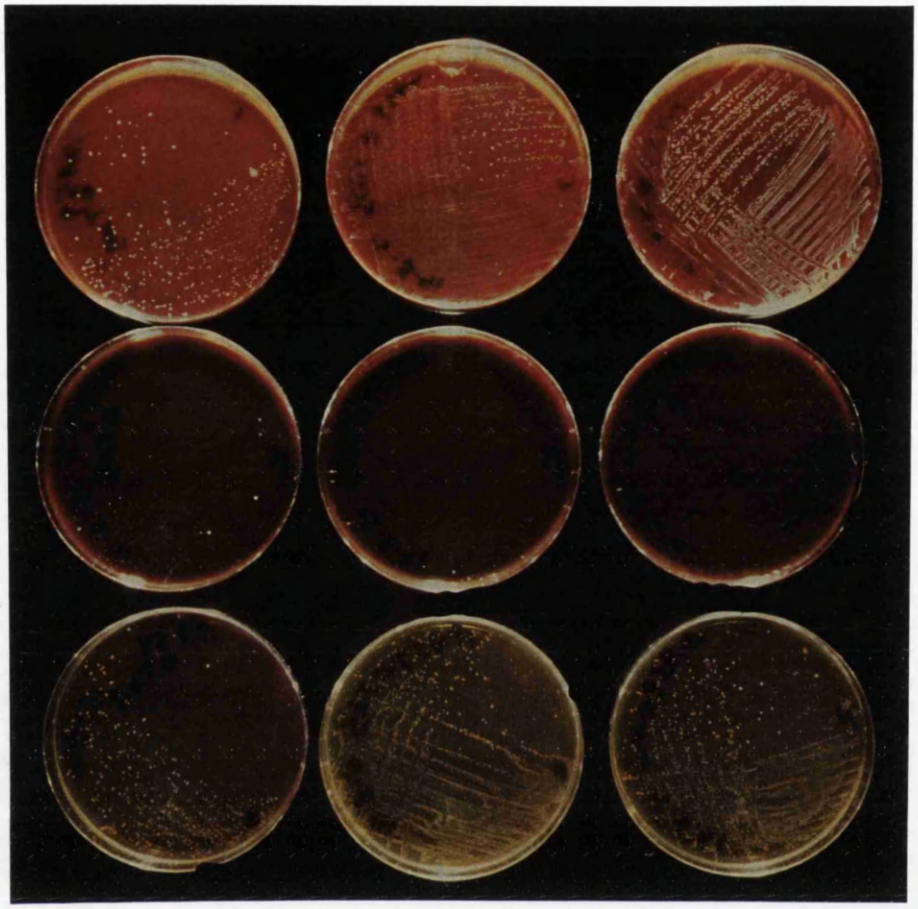


Figure 7 Assessment of three nutrient broths for the growth of *S. uberis*

A - blood agar

B - Edwards agar

C - inulin agar

1 - *S. uberis* preincubated in Todd Hewitt broth

2 - *S. uberis* preincubated in tryptose broth

3 - *S. uberis* preincubated in THTP broth

The API 20 STREP test confirmed that the growth on the three blood agar plates was *S. uberis*. The negative VP (pyruvate substrate) reaction prevented the API test from making any identification of the *S. uberis* grown on Edwards agar. The colonies grown on inulin agar produced negative results in 19 of the 21 reactions, including that of inulin fermentation (figure 8). This finding endorses the manufacturers instructions that only isolates subcultured on blood agar should be used for the API 20 STREP test.

3.11.2 ASSESSMENT OF THE NUMBER OF COLONY FORMING UNITS IN A COLONY OF *STREPTOCOCCUS UBERIS*

One colony of a strain of *S. uberis* grown on blood agar was vortexed in one millilitre of sterile water and five serial one-in-ten dilutions in sterile water were made. Six drops of ten microlitres of each dilution were incubated on blood agar at 37°C for 24 hours.

The first three dilutions produced a field of colonies too numerous and tightly packed together for accurate counting. The next dilution produced circles containing 26, 21, 20, 23, 26, and 26 colonies (average 24 colonies). The next dilution produced a total of 16 colonies from all six drops, and the next dilution one colony only. By calculation, the one original colony contained in the order of 2,500,000 colony forming units.

A

B

C

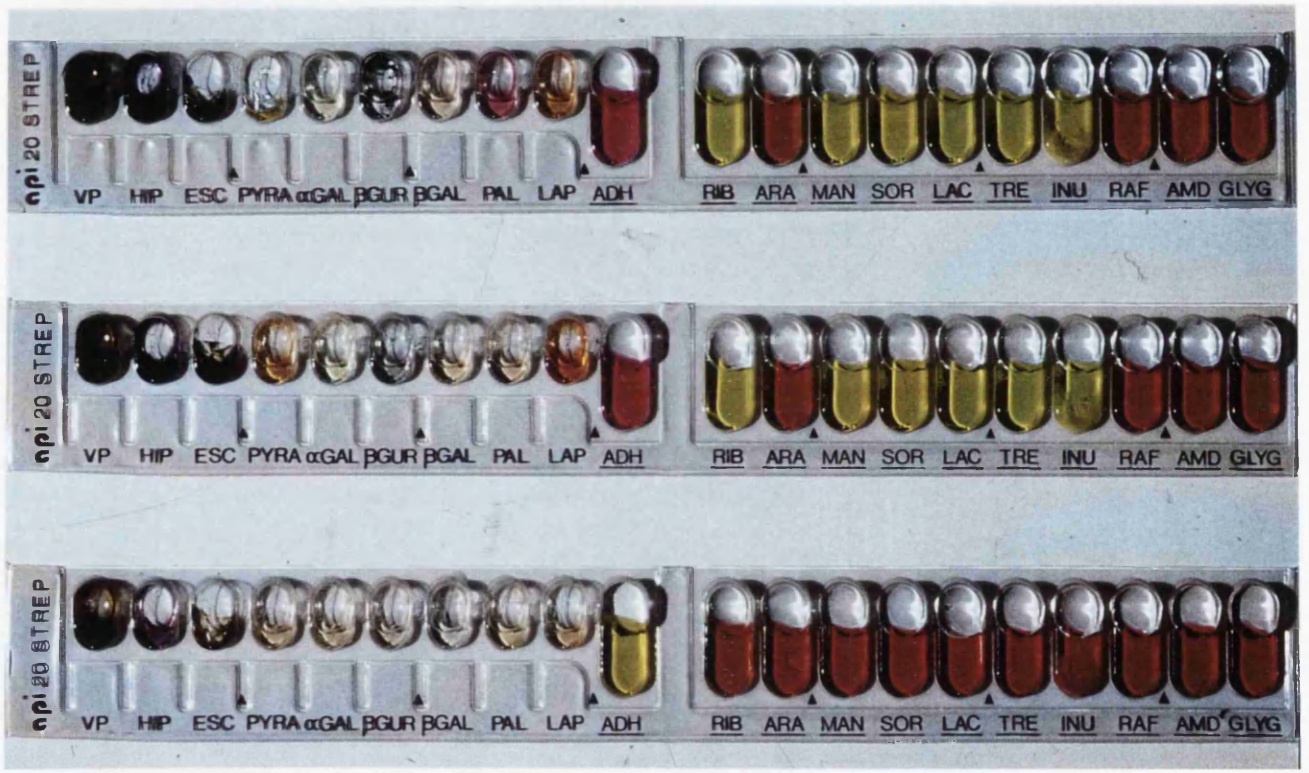


Figure 8 API STREP 20 test

A - typical pattern of results of *S. uberis* grown on blood agar

B - the same isolate of *S. uberis* grown on Edwards agar showing false results in some of the tests on the left side

C - the same isolate of *S. uberis* grown on inulin agar showing more false results of tests including inulin fermentation

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

Since the recognition of *S. uberis* in 1932, mastitis caused by this bacterium continues to be of great importance in the health of dairy cows. Even though intramammary antibacterial treatment is successful, the pathogen's ability to live outside the mammary gland means that it is unlikely that eradication will ever be complete (Cullen, 1966). In order to reduce the ability of the bacterium to cause infection in both the lactating and non-lactating bovine mammary gland, the study of its epidemiology and means of survival outside the mammary gland is paramount. For this to be achieved, simple and rapid tests are required for identification of the bacterium and the different strains that may be encountered.

In 1951, Seeley observed that many of the physiological properties of *S. uberis* were proven but others remained in question, and to this day there is no definitive set of biochemical and/or serological properties by which the species can irrefutably be identified. If, as according to Cullen (1967), the normal biochemical properties of *S. uberis* are taken to include a variable reaction to litmus blue milk but no action on methylene blue milk (whatever its concentration), together with hydrolysis of aesculin and sodium hippurate, and fermentation of inulin, lactose, mannitol, salicin, sorbitol and trehalose, but not raffinose, the question then arises as to whether inulin negative strains (Stantz and Naghski, 1940; Little *et al*, 1946; Seeley, 1951; Garvie and Bramley, 1979), methylene blue milk positive strains (Seeley, 1951), trehalose negative strains (Miller and Heishman, 1940), raffinose positive strains (Little *et al*, 1946; Seeley, 1951; Garvie and Bramley, 1979) and hippurate negative strains (Stantz and

Naghski, 1940; Seeley, 1951; Garvie and Bramley, 1979) should be included in the species. All the above studies detailed the single inconsistencies of the biochemical reactions but, with one exception (Garvie and Bramley, 1979), it was never stated whether particular isolates possessed two or more atypical properties. For instance, one study (Stantz and Naghski, 1940) referred to nine strains of *S. uberis* that were inulin negative, and 15 strains that were hippurate negative, but there was no comment as to whether both negative properties were common to any of the isolates. If the strains exhibited just one of the negative properties, they could be taken to be part of the diversity of the species, but if both negative properties were found in the same isolate, it could be argued that the properties were too diverse for the isolates to be accepted as being *S. uberis*.

Throughout the literature there is a wide spectrum of inconsistency in the identification of *S. uberis*, extreme examples being the belief that the majority of Gram positive and aesculin positive streptococci found in milk samples would be *S. uberis* (Hill and Leigh, 1989), and positive identification as *S. uberis* of streptococcal isolates unable to ferment mannitol and sorbitol, and unable either to ferment inulin and hydrolyse aesculin, or able to ferment inulin only slightly and be unable to hydrolyse sodium hippurate (Garvie and Bramley, 1979).

Unlike other streptococcal species of veterinary importance, *S. uberis* cannot be classified consistently into one of the Lancefield serological groups (Lancefield, 1933). The two other major streptococcal species causing bovine mastitis, *S. agalactiae* and *S. dysgalactiae*, type consistently as Lancefield group B and group C respectively.

Cullen (1967) found between 10.5 and 21% of groups of *S. uberis* isolates (total 745) recovered from milk samples from both clinical and subclinical cases of mastitis and from skin swabs, were of Lancefield group E; all isolates were identified by a number

of tests including fermentation of sorbitol and failure to be able to ferment raffinose. In addition, a proportion of other strains, similarly isolated from the same three sources, that had identical properties other than being either sorbitol negative or raffinose positive, were found to be Lancefield group E positive and, on this finding, he classified these sorbitol negative and raffinose positive strains as being *S. uberis*. However, it is debatable whether such an extrapolation can be relied on when drawn from the small number of strains available; four (28%) of 14 raffinose positive strains and three (33%) of nine sorbitol negative strains being group E positive out of a total of 207 strains isolated from clinical cases. Similarly, the examination of 113 strains from subclinical cases demonstrated that one (17%) of six raffinose positive strains and neither of two sorbitol negative strains were group E positive, and the corresponding figures for 545 strains isolated from skin samples were four (six per cent) of 68 strains and 13 (62%) of 21 strains. Using the same criteria, inulin negative, litmus milk negative, and methylene blue milk positive strains were rejected because no strain showing one of these properties was found to be group E positive (Cullen, 1967).

The other important streptococcal species that has a Lancefield group E serotype is *Streptococcus infrequens* which, although first discovered in milk (Brown, Frost and Shaw, 1926), is more usually isolated from abscesses, for instance, neck abscesses of swine (Diebel *et al*, 1964). It is easily distinguishable from *S. uberis* in being beta haemolytic, rarely alters litmus blue milk, and does not ferment inulin or hydrolyse sodium hippurate (Cullen, 1966).

As yet, it is unknown how the biochemical discrepancies fit in with the mucoid strains of *S. uberis* (Misra and Marshall, 1972), or the division of the species into groups I and II (Garvie and Bramley, 1979) and more recently, into the two separate species of *S. uberis* and *S. parauberis* (Williams and Collins, 1990; 1991). It is to be noted that whereas only four out of 20 group I *S. uberis* isolates (Garvie and Bramley, 1979)

showed inconsistencies, one being inulin negative and three being raffinose positive, only two of the nine isolates classified as group II *S. uberis* showed all the conventional properties; of the other seven isolates, three were raffinose positive, two were inulin negative of which one was also sorbitol negative, and two were sodium hippurate negative of which one only slightly fermented both inulin and sorbitol. At present it is only possible to distinguish *S. parauberis* from *S. uberis* by DNA analysis, and the degree to which *S. parauberis* plays a part in bovine mastitis is unknown. It is possible to speculate that many of the atypical features of *S. uberis* recorded in the past might be attributed to *S. parauberis.*

The epidemiological findings made by past research workers must be considered in the light of the degree of certainty that the organisms stated to be *S. uberis* were, in fact, that particular organism. King (1981) commented that not all authors had defined *S. uberis* in their publications nor had they stated the criteria by which they had differentiated *S. uberis* from other aesculin fermenting streptococci. In an epidemiological survey, in which large numbers of milk and other samples are collected on a regular basis, the protocol used to isolate and identify the organism being studied must be as succinct as possible. Cullen (1966) commented that as final identification depended on extensive biochemical tests, it was necessary to avoid testing too many strains that were not *S. uberis* and, one year later, the same author noted that it was not practicable to carry out an extensive biochemical examination of every organism isolated when screening large numbers of milk samples (Cullen, 1967). The identification of an unknown species of bacteria may require a whole gamut of tests. However, the number of tests needed to isolate and screen for one particular species of bacteria from a known source can be quite small providing the tests are chosen carefully.

It is not known whether all strains of *S. uberis* found on the bovine skin and in the bovine environment can enter the mammary gland and be pathogenic or whether some

strains are limited to a non-pathogenic life outside the mammary gland. With the recent realisation that *S. uberis* was, in fact, two species and the division of the species into *S. uberis* and *S. parauberis* raises questions as to whether the two species have similar or different ecological niches and, if the latter is the case, whether *S. parauberis* is as pathogenic as *S. uberis* or just a commensal organism living in the environment and on the bovine skin. Whether or not the latter is the case, the two species will have to be able to be distinguished for an epidemiological study of *S. uberis* to be of any value. The possibility of *S. parauberis* being a harmless inhabitant of the bovine skin raises the speculation of using it to inhabit udder and teat skin to such an extent that *S. uberis* was not able to colonise around the teat orifice and gain entrance to the mammary tissue.

Without having a herd with a known endemic *S. uberis* problem to sample it was difficult to asses the development of the *S. uberis* isolation protocol evolved during this study. Despite extensive testing of milk and other samples from seven dairy herds, only three isolates of *S. uberis* were collected from milk samples and no isolates were collected from skin, vaginal or rectal swabs. There is no way of knowing whether the herds with a high milk somatic cell count were infected with *S. uberis* in addition to the very obvious *Staph. aureus* infection encountered on four of the farms. The low isolation rate of *S. uberis* could be a reflection of the low incidence rate of *S. uberis* in Scotland as found by Logue *et al* (1993) who reported that whilst the isolation rate of *Staph. aureus*, *S. agalactiae*, *S. dysgalactia* and *E. coli* were all slightly higher in Scotland for the period 1980 to 1992 when compared with the figures for the whole of Great Britain, the isolation rate of *S. uberis* in Scotland was little more than half that of Great Britain. It could also be presumed that the high immune response involved in trying to contain the *Staph. aureus* infection was sufficient to control any *S. uberis* present, and only when the *Staph. aureus* had been eradicated would a potential *S. uberis* problem become apparent.

In addition to *S. uberis*, the species of streptococci that could be found in bovine milk or in the environment of dairy cows and which needed to be eliminated during the testing procedure were *S. pyogenes*, *S. agalactiae*, *S. dysgalactiae*, *S. bovis*, *L. lactis* (*S. lactis*), *S. faecalis*, *S. faecium* and *A. viridans*. The four tests used in this study, namely inulin fermentation, aesculin and sodium hippurate hydrolysis, and the lack of beta haemolysis of blood agar distinguished *S. uberis* from all the above bacterial species as well as ensuring the elimination of other streptococcal species of animal importance, such as *S. infrequens*, *S. zooepidemicus*, *S. equi*, *S. equinus*, *S. suis*, *S. pneumoniae*, *S. porcinus* and *S. avium*, that may be found in a farm environment. The four tests chosen always required a positive reaction to the substrate by the isolate thus eliminating the possibility of false negative results and, with the exception of the hippurate test which was always the final test, all the tests were carried out on agar medium thus allowing single colonies to be harvested for subculture, thus reducing the possibility of a contaminant organism being cultured with the test isolate in a broth medium.

It was decided that the ability to ferment inulin, as stated by Cullen (1967), Buxton and Fraser (1977) and Carter and Cole (1990), was a pre-eminent property of *S. uberis* despite claims that certain *S. uberis* strains existed that did not ferment inulin (*vide supra*), although both Little (1940) and Cullen (1967) found that inulin negative isolates of *S. uberis* were nearly always atypical in some other respect. The more recent API 20 STREP Analytical Profile Index (bio Mérieux, 1990) recorded 17 of the 116 test results, which identified a bacterium as *S. uberis*, as being inulin negative; 15 of the 17 were given an identification confidence rating of between 97 and 100%, one had a confidence rating of 83% and the other 32%.

Of the streptococcal species that are of importance in animals only *S. uberis*, *S. bovis*, *S. equinus* and *S. pneumoniae* are inulin positive, with *S. suis* being partially positive and *S. agalactiae*, *S. dysgalactiae* and *S. faecalis* inulin negative (Carter and Cole,

1990). Buxton and Fraser (1977) stated that of the streptococci that could be isolated from bovine milk only *S. uberis* and *S. bovis* were inulin positive, with *L. lactis* (*S. lactis*) being an important addition to the aforementioned inulin negative species. Although *S. faecium* is also ruled out by the inulin test, there is a subspecies of the organism *S. faecium var. casseliflavus* that can ferment inulin (Mundt, 1968b). This organism can be distinguished from *S. uberis* by other tests in the API 20 STREP system, but for large numbers of potential *S. uberis* isolates this would prove expensive. However, the two organisms can be distinguished by the fact that *S. faecium var. casseliflavus* grows on blood agar as a bright yellow colony. *Streptococcus faecium* and its subspecies are in group D of the serological classification of Lancefield (1933), a group to which *S. uberis* has not been found to belong, however differentiation by serological typing would not be practicable if many samples were to be analysed on a routine basis.

It is accepted that *S. uberis* hydrolyses aesculin (Cowan and Steele, 1974; Buxton and Fraser, 1977; Carter and Cole, 1990) and, of the streptococcal species associated with the Bovidae, *S. pyogenes*, *S. agalactiae* and *S. dysgalactiae* do not hydrolyse aesculin. At the start of this study, aesculin hydrolysis was used as the primary screening test before it was found that many and various organisms grew well on Edwards agar, to the detriment of the growth of *S. uberis* and the isolation of discrete colonies. The inulin fermentation test replaced aesculin hydrolysis as the primary screening test but the latter was retained in the test protocol as it allowed *A. viridans*, which also ferments inulin but grows as large black colonies on Edwards agar, to be easily distinguished from *S. uberis*, which grows as tiny colonies and caused the medium to become transparent.

Forty-eight hour growth on blood agar distinguished the colonies of streptococcal species from other bacterial species including the larger and more colourful colonies of

the staphylococcal species and *S. faecium var. casseliflavus*: The type of haemolysis surrounding the streptococci colonies was also visible.

Both *S. agalactiae* and *S. uberis* hydrolyse sodium hippurate (Cowan and Steele, 1974; Buxton and Fraser, 1977; Carter and Cole, 1990) as do some strains of *S. dysgalactiae* (Cowan and Steele, 1974), some strains of *S. faecalis* (Cowan and Steele, 1974; Carter and Cole, 1990), *S. faecium* (Cowan and Steele, 1974), some strains of *L. lactis* (*S. lactis*) (Buxton and Fraser, 1977), *A. viridans* (Cowan and Steele, 1974) and some strains of *S. avium* (Carter and Cole, 1990). All these species were distinguished from *S. uberis* by the inulin and/or aesculin tests and so were eliminated in the early stages of the isolation procedure. However, the sodium hippurate test was essential to distinguish *S. uberis* from *S. bovis*, the latter having many similar properties to *S. uberis* including inulin fermentation and aesculin hydrolysis but not the ability to hydrolyse hippurate.

From the small number of isolates recovered from milk samples collected during the course of this study, those that gave the correct response to the four tests were confirmed as being *S. uberis* by the API Strep 20 test. Occasionally an isolate that gave the wrong response to one of the tests was also tested by the API Strep test; no such isolate was ever confirmed as being *S. uberis.*

In the present study, *S. uberis* was not isolated from samples collected in the months of March, April and May from Farm 1 but this could have been due to the isolation procedures used at that time. The three isolates of *S. uberis* from Farm 1 cultured from milk samples collected in the months of August and September reflected the improved isolation procedures adopted during the course of the study. This finding is in contrast to that of another study (Cullen, 1966) in which *S. uberis* was isolated from bovine milk samples in all months of the year except for the months of July, August and September.

Four of the seven herds investigated in this study were shown to have an endemic *Staph. aureus* infection. The lack of any investigation of previous clinical cases together with comprehensive antibacterial susceptibility testing was surprising, as was the apparent failure to use the SMRA somatic cell count information, gathered at some expense to the farmer, to realise the extent of the subclinical disease with its inherent reduction in quantity and quality of milk produced, and consequential loss of revenue. Most strains of *Staph. aureus* tested for antibacterial susceptibility were resistant to penicillin alone, or in combination with streptomycin, with which most infected cows had been routinely treated. The only antibacterial agent to which nearly all isolated strains of the pathogen were susceptible was the amoxycillin and clavulanic acid combination ("Synulox", Pfizer). However, occasional strains of *Staph. aureus* were isolated that were resistant to many of the antibacterial agents tested including amoxycillin and only moderately susceptible to the amoxycillin and clavulanic acid combination, which emphasised the need for antibacterial susceptibility testing to be one of the diagnostic tools used when investigating problem herds. The high somatic cell counts in these herds reflected the high level of subclinical infection throughout the herds, especially in the older cows.

In the herds investigated, the *Staph. aureus* infection was of a much greater significance to the veterinary and economic health of the herd than any level of *S. uberis* encountered at the time of sampling. Edwards (1933), who also experienced the problems caused by colonies of staphylococci and *B. coli* growing on culture plates, commented that if they were present in large enough numbers, they would obscure the growth of any streptococcal colonies present, as well as preventing streptococcal colonies being distinguished from those of non-haemolytic staphylococci. The same problem could well have been experienced in this study, although only when the *Staph. aureus* problem was eradicated would this be known. Possibly the results of past published surveys of the prevalence of *S. uberis* infection in dairy herds have been affected similarly.

The use of enrichment media to boost the recovery of a weak or sparsely present isolate has been well documented. Davis and Capps (1914) observed that pure cultures of streptococci (no species identified) incubated at 37°C in unpasteurized milk multiplied nearly 70 fold within 22 hours but died back to only about twice the original number by 48 hours, whereas other bacteria in the milk multiplied very slowly in the first 22 hours but increased markedly by 48 hours. For the initial step in the recovery of *S. agalactiae* from contaminated sites outside the bovine udder, Chodkowski and Lancaster (1949) found that sterilised milk alone was the best medium when the ratio of contaminants to *S. agalactiae* was low, but sterilised milk containing the dyes potassium tellurite and boric acid was more suitable when the levels of contaminants was higher, even though the dyes had an inhibitory effect on the growth of *S. agalactiae*. The latter medium was also found suitable for the recovery of *S. uberis* (Cullen, 1966) as was sterile milk, colony counts from control cultures always being higher after incubation in sterile milk than in infusion broth.

The recovery of *S. uberis* from swab samples taken from various bovine sites, skin (Bramley *et al*, 1979), rectum and vulva (Bramley *et al*, 1979; Kruze and Bramley, 1982), faeces and straw bedding (Bramley, 1982), and skin swabs (Buddle *et al*, 1988) was greatly enhanced when the samples were preincubated in litmus milk before subculture on various differential agar media.

Sharma and Packer (1970) incubated milk and skin swab samples in tryptose broth inoculating the broth cultures on differentiating agar media. Following incubation, colonies from the agar media were reincubated in the tryptose broth before being subjected to further substrate tests. Hill and Leigh (1989) used Todd Hewitt broth to culture isolates of *S. uberis* for DNA analysis.

In this study, the initial isolation procedure involved sub-culturing a milk or swab sample on one of several types of agar, with or without initial incubation in Todd Hewitt broth (Todd and Hewitt, 1932), tryptose broth (Sharma and Packer, 1970) or THTP broth (a mixture consisting of equal volumes of Todd Hewitt broth and tryptose broth). All three broths encouraged growth of streptococcal species but they did nothing to reduce the contaminating organisms that subsequently grew on the agar plates following the subculturing of broth-incubated milk samples from the Farm 7 herd, and skin, vaginal and faecal swab samples from other herds. However it was very satisfying to find that the simple expedient of mixing equal volumes of Todd Hewitt broth and tryptose broth (THTP broth) produced a greatly increased growth of *S. uberis* than that produced by either constituent broth alone.

Streptococcus uberis colonies have been successfully sub-cultured directly from inulin agar to Edwards agar without an intervening broth stage (Bramley *et al*, 1979; Bramley, 1982). In the present study it was found that direct culturing of colonies from both inulin and aesculin agars weakened the colony growth in subsequent subculturing, and thus put doubt on the validity of the results of subsequent tests using those particular cultures. The literature accompanying the API 20 STREP test kits emphasises that only cultures grown on blood agar should be tested. In this study, API 20 STREP testing of *S. uberis* cultures grown on aesculin or inulin agar produced atypical reactions to many of the tests and threw doubt on whether a bacterial colony grown on a single substrate medium is able to adapt its metabolism successfully enough to utilise a different single substrate medium on which it is subcultured. Rather than putting a particular isolate through a series of successive tests, the isolation and identification may be better carried out by subjecting colonies of a particular isolate to a set of tests run in parallel, or, if a series of tests on the same culture is required, to incorporate an enrichment stage between each single substrate test.

Unfortunately literature on the use of litmus milk as an enrichment medium was not found or appreciated until the practical part of the study was drawing to a close. Before further work was carried out into the presence of *S. uberis* in dairy herds, it would be of great benefit to carry out a preliminary study into the best nutritive and enriching medium, or combinations of media, for *S. uberis* together with the definitive incubation time for the maximum multiplication in order to ensure the best possible chance of *S. uberis* being isolated if it is present in the sample.

The recent method of subtyping *S. uberis* by DNA amplification (Jayarao *et al*, 1992) with its ability to differentiate a greater number of subtypes than by the restriction endonuclease method (Hill and Leigh, 1989) should be investigated, consideration being given to the ease of application and the time necessary for the procedure, bearing in mind the large number of isolates of *S. uberis* that would be collected during the course of an epidemiological study.

It was hoped that the offer of a free and comprehensive mastitis investigation with antibacterial susceptibility testing would generate a good response from farms in the south-west of Scotland, from which herds could be chosen to be surveyed for *S. uberis* infection. That so few herds with a mastitis problem were put forward by all the veterinary practices contacted was both surprising and disappointing. Of the five farms that were forwarded from veterinary practices, three had low milk somatic cell counts and correspondingly little mastitis problem, one had experienced two cases of clinical *S. uberis* mastitis in the recent past, which had been identified by one of the Veterinary Investigation Centres, and one had a mastitis problem that, according to the farmer, was intractable to standard veterinary treatment, and was therefore being treated with homeopathic remedies. The other two farms were already known to the Medicine Department of Glasgow University Veterinary School, one having a persistent high somatic cell count problem, and the other having a calf neonatal disease problem with a

high mortality rate. The mastitis problems encountered on the farms visited reflected the variation in housing systems and herd management seen on farms in the United Kingdom at the present time.

All the farmers visited realised the impact that financial penalties for producing milk with high somatic cell counts would make to their livelihood besides the other milk quality controls (total bacterial count and antibiotic residues) already in place. At the time of the study the Scottish Milk Marketing Boards were imposing a stricter penalty regime than the Milk Marketing Board of England and Wales; in 1993 the penalties for England and Wales were adjusted to that of Scotland. It is to be hoped that with recent European community directives on better quality milk production, more farmers would have a greater awareness of the need for higher standards in mastitis control and prevention.

Much time and effort is now being spent in developing mastitis vaccines. As a wide number of individual pathogens can cause mastitis, some workers think that a single multivalent vaccine will never exist (Hillerton, 1994) and, as there are many different strains of the major mastitis pathogens, it is possible that individual vaccines may have to be made according to the strains of pathogen found on an individual farm.

Parenteral vaccination does not lead to improvement in the components of the immune defences of the udder, these only being attracted into the udder once the site of infection is well established (Colditz and Watson, 1985). Local immunisation of the mammary gland against mastitis pathogens has been shown to be effective in increasing the immune protection in the gland, but this has to be done during the dry period as infusion into lactating glands may result in a poor response presumably due to the antigen being flushed out. However such dry period infusions cause a loss of milk yield during the subsequent lactation (Watson, 1981), as well as disturbing the keratin plug in the streak

canal and increasing the possibility of environmental pathogens being inadvertently pushed into the gland during the immunising procedure.

It is unlikely that eradication will be possible for the environmental pathogens as there will always be new challenges from sources that cannot be controlled and from pathogens that may possibly be able to mutate sufficiently to render ineffective a particular strain vaccine. However vaccines may have a role in increasing resistance to a specific pathogen at a particular time in the lactation cycle, for example, vaccination against *E. coli* at around the time of parturition and early lactation, and against *S. uberis* during the dry period. In view of evidence showing that, on average, between 40 and 50% of all dairy cows never contract mastitis whilst 25% of the herd suffer mastitis at least once every lactation (Hillerton, 1993), vaccination would be better carried out on an individual cow basis rather than a herd basis.

Perhaps the need for more and more ways of counteracting mastitis are being fuelled by the way that the cow is being made to adapt to modern milking methods rather than adapt modern milking methods to harness the natural resistance to mastitis in the cow. It has been known for nearly half a century that slow milking cows have a lower incidence of mastitis than their faster milking colleagues and that teat canal diameter governs the speed of milking (Dodd and Neave, 1951). Other studies have shown that the thickness of keratin in the teat canal can vary widely (McDonald, 1970). Unfortunately selecting cows for slowness of milking runs contrary to the modern practice of batch milking a group of cows in the ubiquitous herringbone parlour where a slow milking cow that rarely suffers from mastitis is far more likely to be culled than her faster milking, more mastitis susceptible colleagues. This is unlikely to be a problem in an abreast parlour, or a tandem parlour with each stall having its own entrance and exit, where each cow is milked on an individual basis.

Field studies of the epidemiology of *S. uberis* in dairy herds remain to be carried out. It is considered that only when such work is done can the questions about the epidemiology and pathogenicity of the various strains of *S. uberis*, posed at the beginning of this study, be answered. In the light of one nationwide epidemiological survey (Wilson and Richards, 1980) which showed that the prevalence of *S. uberis* was over two and a half times higher in southern and the far south-west of England than in Scotland, further studies may well be more profitably carried out in the former than the latter part of Great Britain.

An epidemiological study cannot be undertaken lightly considering the vast number of milk and other samples to be processed at frequent intervals. Buddle *et al* (1988), using the bacteriocin-like inhibition typing method of Tagg and Vugler (1986), were able to recover several different strains of *S. uberis* from one skin swab, and this raises the possibility of different strains being present in milk samples and, therefore, on the initial differential agar plate. It would be impractical to individually subculture all the potential *S. uberis* colonies that appear on the initial agar plate and perform a DNA analysis on all the isolates of *S. uberis* recovered but, without doing so, there is no certainty of identifying all the different strains of *S. uberis* present. For instance, if there are 100 individual colonies on the initial differential agar plate, how many of them should be subcultured? To attempt to answer this question, a exhaustive preliminary study would need to be carried out on a small number of milk samples from known infected cows in the herd being investigated.

Appendix I Tests used to Identify S. uberis

Research Paper	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Number of isolates	3	33	?	14	25	141	83	14	24	8	?	52	?	114	575	1481	172	175	2	29	495	31	25	?	?	70	?
Haemolysis of blood agar	γ/ν	−β	...	...	ν	α/γ	α	γ	ν	...	(α/γ)	γ/ν	...	...	...	α/γ	...	...	β	...	...	...	...	...	α/γ	...	...
Methylene blue milk																											
concentration 1: 1,000	...	...	...	...	...	...	...	...	...	...	...	1/52	...	...	-	-	...	2/160	-	...	...	...	...	...	...	...	...
1: 5,000	...	...	...	(R)	...	var	...	...	CR	...	var	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1:10,000	R	...	...	...	...	...	...	var	...	...	...	...	...	...	-R	...	...	49/50	+	...	...	...	...	...	...	...	...
1:15,000	...	...	...	...	...	...	...	...	...	R(C)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1:20,000	...	R(C)	...	...	...	...	R	...	...	...	R	...	...	...	...	...	...	160/160	...	...	...	...	...	...	...	...	...
no concentration given	...	...	R(t)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Litmus milk	A	var	A	var	CR	A	var	var	...	ACR	AC	A(CR)	...	...	var	var	...	...	...	A(R)	...	...	...	...	...	...	...
Hydrolysis of aesculin	...	-*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	...	+	+	+	+	+	+	+	+	+	+
Hydrolysis of arginine	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	+	...	174/175	+	+	...	+	...	...	+	...	+
Hydrolysis of hippurate	+	+	+	+	+	+(-)	68/83	+	+	+	+	+	+	...	+	+	+	174/175	+	27/29	+	...	...	...	...	?+	+
Hydrolysis of starch	...	...	...	...	...	...	...	...	...	3/8	...	11/52	...	...	...	var	...	...	...	...	...	...	...	...	-	...	...
Fermentation of																											
Amygdalan	...	...	...	...	19/19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Arabinose	...	...	...	-	...	-	...	-	...	...	-	-	...	...	...	...	...	1/175	-	...	...	...	...	...	...	...	...
Cellobiose	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	10/10	+	...	...	...	...	...	...	...	...
Dulcitol	...	...	...	-	...	...	...	...	...	...	...	1/11	...	...	...	...	...	3/30	...	...	...	...	...	...	...	...	...
Fructose	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Galactose	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	10/10	...	...	...	...	...	...	...	...	...
Glucose/Dextrose	4.6	<4.8	4.7	<5.0	4/4	...	...	...	<4.9	<4.9	<5.0	<4.9	...	...	...	...	...	...	<5.3	...	...	...	...	...	...	...	...
Glycerol/Glycerine	...	...	-	-	0/4	...	...	...	...	...	...	+(a)	...	...	...	...	...	0/30(a)	...	...	...	...	...	...	...	...	...
Inulin	4.6	+	+	+	19/19	+(-)	74/83	+	+	+	+	43/52	+	...	+	+	+	158/175	+	26/29	+	+	+	+	+	+	+
Lactose	4.5	+	+	...	4/4	+	...	4.5	+	+	+	+	...	...	...	+	...	...	+	...	...	...	...	...	...	...	...
Levulose	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Maltose	...	...	...	+	...	...	...	...	+	...	+	+	...	...	...	...	...	10/10	+	...	...	...	...	...	...	...	...
Mannite	4.6	+	+	...	...	...	...	+	+	+	...	...	+	...	...	...	+	...	...	...	...	...	...	...	...	...	...
Mannitol	...	...	...	+	+	+	+	...	...	...	+	+	...	...	+	+	...	+	...	+	...	...	...	...	+	?+	...
Mannose	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	10/10	+	...	...	...	...	...	...	...	...
Melibiose	...	...	...	...	...	...	...	...	...	...	...	0/11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Raffinose	-	-	-	-	0/19	-	-	-	...	-	-	1/52	-	...	-(+)	-(+)	-	12/175	-	6/29	-	-	...	-	-	-	-
Saccharose	4.6	+	+	...	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Salicin	4.6	+	+	+	19/19	...	+	+	+	...	+	+	...	...	+	+	+	10/10	+	+	...	...	...	...	+	?+	...
Sorbitol	...	...	...	+	19/19	+	...	+	+	...	+	+	...	...	+(-)	+(-)	+	172/175	+	28/29	...	...	...	...	+	+	...
Sucrose	...	...	...	+	...	...	...	...	...	...	+	+	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...
Trehalose	...	...	...	+	19/19	+	...	?/14	+	...	+	+	...	...	+	+	...	+	+	+	...	...	...	...	+	...	...
Xylose	...	...	...	-	0/19	...	...	...	...	...	...	-	...	...	...	...	...	0/20	-	...	...	...	...	...	...	...	...
Growth at 10°C	...	...	...	...	0/4	...	10/70	...	...	...	...	51/52	...	...	...	...	...	160/160	...	...	...	...	...	...	+	...	...
Growth at 45°C	...	...	...	...	?/4	...	60/70	...	...	5/8	...	50/52	...	...	...	...	...	9/175	...	...	...	...	...	...	?+	...	...
Growth at 50°C	...	...	...	...	0/4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Viable after 30 min at 50°C	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Viable after 30 min at 60°C	...	-	...	...	...	...	0/70	...	-	-	...	42/52	...	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Gas from acid citrate	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	-	-	...	...	-	...	-
Lancefield group E positive	...	...	...	...	...	...	...	...	...	...	...	...	-(+)	16-21%	6-62%	10-21%	...	37%	...	...	...	...	...	...	...	...	...
10% Bile salt tolerance	...	...	...	...	...	...	...	...	...	...	...	11/11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
40% Bile salt tolerance	...	...	...	...	...	...	...	...	1/24	...	...	5/11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

Key - See next page

Appendix I Tests used to Identify S. uberis (concluded)

Key

1	Ayers & Mudge (1922)	10	Francis (1942)	19	Misra & Marshall (1972)
2	Edwards (1932)	11	Little et al (1946)	20	Garvie & Bramley (1979)
3	Diernhofer (1932)	12	Seeley (1951)	21	Bramley et al (1979)
4	Plastridge et al (1934)	13	Hughes (1960)	22	Bramley (1982)
5	Ferguson (1938)	14	Sweeney (1964)	23	Kruze & Bramley (1982)
6	Plastridge & Williams (1939)	15	Cullen (1966)	24	Robinson et al (1983, 1985)
7	Slantz & Naghski (1940)	16	Cullen (1967)	25	Bramley (1984)
8	Miller & Heishman (1940)	17	Sharma & Packer (1970)	26	Buddle et al (1988)
9	Little (1940)	18	Roguinsky (1971)	27	Hill & Leigh (1989)

+	= All isolates positive	-R	= No reduction
-	= All isolates negative	(CR)	= Variable coagulation & reduction
+(-)	= Isolates usually positive	(R)	= Variable reduction
-(+)	= Isolates usually negative	(a)	= Aerobic conditions only
α	= Alpha haemolysis	(t)	= Temporarily
β	= Beta haemolysis	/	= Proportion positive of those tested
$-\beta$	= Not beta haemolysis	<	= Less than
γ	= Gamma haemolysis	var	= Various results
ν	= Viridan haemolysis	?	= Number unspecified
(α)	= Weak alpha haemolysis	?+	= Number unspecified positive
(α/γ)	= Usually alpha or gamma haemolysis	*	= By the method of Harrison and van der Leck (1909a,b)
A	= Acidification	4.6 etc	= limiting pH
C	= Coagulation	...	= not recorded
R	= Reduction		

Appendix II Farm Information Sheet

STREPTOCOCCUS UBERIS FARM INFORMATION

OWNER'S NAME .....
ADDRESS .....
TELEPHONE NO. ..... Herdsman's Tel.No.....
Farm size ..... Years/months .....
Other enterprises .....
Herd size ..... No. 1st calf heifers .....
Breed ..... Av.M.Y. ..... Quality band .....
Identification - Name/number/tag/collar/freeze brand
Source of cows - Home bred/bought in/market

Housing - Cubicles/kennels/byre/yard/other
Bedding material ..... Clean/dry
Exercise area/passages cleaned ..... X's daily

Parlour type ..... Age .....
When last inspected ..... All/most/some faults corrected
Circulation cleaner type ..... Time ..... Temp.....
Teat washing - Bucket/hose/disinfectant Type ..... Gloves
Teat drying - Cloth/paper/common/individual
Foremilk exam. - Floor/cup/boot/every 1/4 /milking
No.cows milked per unit per hour .....
Post milking teat dip/spray type .....
Post milking standing time .....

Winter feed ..... METHOD .....

A.I./bull ..... Age at 1st calving .....
Calving pattern ..... Index .....
Calving - 1st service interval ..... % turn .....
Drying off - Sudden/once daily Criteria Yield/time
Length dry period ..... Calving facilities .....

Milk recorded ..... TBC ..... SCC .....
No.cases clin.mastitis in last week (2%) ..... Per year .....
Milk sampled pre-treatment - Bacteriology .....
Treated promptly ..... M.C.tubes per year ..... Type .....
D.C.tubes ..... Fly control tags/spray
Cull rate ..... Av.age ..... Reasons ..... By records

TRB 91/1/B38

Appendix III Summary of Farm and Herd Management

Farm	1	2	3	4	5	6	7
Location	N.W. Glasgow	Lanarkshire	Lanarkshire	S.Ayrshire	Lanarkshire	Lanarkshire	Dumfriesshire
Size (hectares)	100	200	40	80	140	90	160
Other Enterprises	Sheep, pigs, horses, deer	None	Sheep	None	Sheep, cereals	Beef, sheep	Cereals
Number of Cows	90	235	50	120	120	60	120
Breed of Cows	Friesian	Jersey	Friesian	Friesian	Friesian	Friesian	Friesian
Winter Housing	Cubicles	Cubicles/yard	Cubicles	Cubicles	Cubicles	Cubicles	Cubicles
Bedding Material	Sawdust	Sawdust/straw	Straw	Sawdust	Sawdust	Sawdust	Sawdust
Calving Pattern	All year	All year	Spring	All year	July-Sept	July-Aug	All year
Pre-milking Routine	Superdip (Iosan) hose	None	Super Iodip (Deosan) cloth	Super Iodip (Deosan) hose	Blue Towel (Genus) wipe	Teat Dip (Iosan) hose	Super Iodip (Deosan) hose
Post-milking Routine	Superdip (Iosan) spray	Super Ex-cel (Deosan) spray	Super Iodip (Deosan) teat dip	Super Iodip (Deosan) spray	None	Super Iodip (Deosan) teat dip	Super Iodip (Deosan) teat dip
Milking Cow Therapy	Myelipen QR	Streptopen MC	Streptopen MC Leo Yellow	Synulox	Tetra Delta	Streptopen MC	Streptopen MC
Dry Cow Therapy	Myelipen DC	Streptopen DC	Streptopen DC	Leo Red	Leo Red Orbenin DC	Streptopen DC	Streptopen DC
SMRS Recorded	Yes	Yes	Yes	No	Yes	Yes	Yes
Reason for Referral	High milk SCC	High milk SCC	High milk SCC	Clinical cases	High milk SCC	MSCC <250,000 but rising	MSCC >250,000
Main Pathogen Isolated	Staphylococci	Staphylococci	Staphylococci	Staphylococci	Staphylococci	None	None

Appendix IV Summary of Laboratory Techniques Carried Out on Milk and Other Samples Collected from Farms

Farm	Visit	Date of Sampling	Number of Cows Sampled	Source of Sample	Number of Samples	Initial Procedure	Further Procedures
1	1	18.03.91	9	Quarter	36	Edwards agar	Aesculin hydrolysing colonies subcultured on blood agar and in 5ml Todd Hewitt Broth Todd Hewitt Broth positive, non beta haemolytic colonies subcultured from blood agar to inulin broth
				Vagina	9	As milk samples	As milk samples
				Rectum	9	As milk samples	As milk samples
1	2	22.04.91	15	Quarter	59	Blood agar	Streptococcal colonies subcultured on Edwards agar Antimicrobial tests on Staphylococcal colonies
1	3	13.05.91	10	Quarter	40	Blood agar	Analysis of Staphylococcal growth
1	4	29.08.91	67	Jar	67	THTP broth	Broth growth subcultured on Inulin agar. Inulin fermenting colonies subcultured in THTP broth Broth growth subcultured on blood agar and Edwards agar Aesculin hydrolysing, non beta haemolytic isolates on blood agar subcultured in sodium hippurate broth
1	5	11.09.91	70	Jar	70	THTP broth	Broth growth subcultured on inulin agar. Inulin fermenting colonies subcultured in THTP broth then on Edwards agar
				Quarter	8		Aesculin hydrolysing colonies subcultured on blood agar Non beta haemolytic colonies subcultured in sodium hippurate broth
2	1	15.04.91	20	Quarter	79	Blood agar	Streptococcal colonies subcultured on Edwards agar
2	2	01.05.91	9	Quarter	19	Blood agar	Antimicrobial tests on Staphylococcal colonies
3		20.05.91	10	Quarter	35	Blood agar Edwards agar	Antimicrobial tests on Staphylococcal colonies Aesculin hydrolysing colonies subcultured in inulin broth
4		01.07.91	43	Quarter	169	Blood agar	Streptococcal colonies subcultured on Edwards agar Antimicrobial tests on Staphylococcal colonies
5		16.07.91	101	Jar	101	Blood agar Todd Hewitt Broth	Antimicrobial tests on Staphylococcal colonies Broth growth subcultured on Edwards agar. Aesculin hydrolysing colonies subcultured in Todd Hewitt broth Broth growth subcultured in inulin broth and on inulin serum agar. Inulin fermenting colonies on inulin serum agar subcultured in Todd Hewitt broth then subcultured in sodium hippurate broth
				Skin	101	Todd Hewitt Broth	Broth growth subcultured on Edwards agar, then as milk samples
6		30.07.91	39	Jar	39	Blood agar Todd Hewitt Broth	Antimicrobial tests on Staphylococcal colonies Broth growth subcultured on Inulin serum agar and Inulin agar Inulin fermenting colonies subcultured on Edwards agar
7		29.10.91	114	Jar	114	Inulin agar THTP broth	No growth Broth growth subcultured on inulin agar (with and without thallium acetate) Inulin fermenting colonies subcultured on blood agar Non haemolytic colonies subcultured on Edwards agar

Appendix V Farm 1 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml)

Cow No.	07.08.89	28.08.89	27.09.89	16.03.90	26.02.91	22.03.91	24.04.91
1	-	37	5	35	-	-	-
2	92	2	20	39	36	31	27
3	21	45	47	103	115	-	-
4	224	681	-	31	50	69	73
5	55	36	99	-	-	383 (S2)	28
6	339	62	33	2734 (C)	-	-	-
6	-	-	-	-	198	95	72
7	82	49	61	189	79	67	120
8	38	19	16	242	293	436 (S2)	-
9	51	569	631	178	-	62	35
10	211	48	42	1248	183	405 (S2)	1256 (S3)
11	26	71	25	1465	-	-	-
12	35	26	35	-	973 (S1)	2542 (S2)	926
13	1913	95	32	-	-	-	-
14	26	277	-	53	218	2705 (S2)	616 (S3)
15	162	613	156	163	-	-	-
16	99	68	-	81	1530 (S1)	1087 (S2)	1435 (S3)
17	12	64	96	162	-	-	-
18	-	-	-	-	51	670 (S2)	628
19	51	47	52	41	-	-	-
20	16	24	32	-	-	-	-
21	22	14	2	102	50	61	51
22	102	206	165	214	316 (S1)	284 (S2)	182
23	34	23	53	-	-	-	-
24	7	65	65	-	-	-	-
25	48	162	54	-	-	213	221
26	808	659	736	-	102	88	88
27	306	154	-	-	46	41	36
28	2	93	87	-	-	-	-
29	133	66	45	119	79	115	87
30	69	67	988	54	-	-	-
31	395	128	253	-	296	379	606 (S3)
32	-	-	27	-	51	132	35
33	56	99	32	135	92	100	500
34	26	95	51	19	335 (S1)	844 (S2)	916 (S3)
35	124	2481	3352	-	-	-	-
36	69	342	106	178	-	-	-
37	2	24	14	5	81	99	-
38	22	17	12	39	61	68	-
39	31	32	38	-	110	144	602 (S3)
40	312	3153	-	-	-	-	-
41	65	89	49	148	-	-	38
42	21	33	22	37	-	-	-
43	653	281	125	681 (C)	-	-	-
43	-	-	-	-	478 (CL)	-	-
44	35	3416	-	-	-	-	-
45	-	-	236	697	-	-	-
46	9	133	13	68	-	-	-
47	68	41	68	153	1126 (S1)	1754 (S2)	3082 (S3)
48	-	-	3236	674	-	-	-
49	-	-	4	547	-	-	-
50	42	58	87	-	-	-	76
51	-	-	-	438	-	132	75
52	174	33	25	-	185	253 (S2)	142
53	27	28	37	83	85.000	390 (S2)	91
54	-	-	-	394	78	44	33
55	124	386	113	3	-	-	-
56	132	647	-	-	-	-	34
57	-	-	-	35	-	-	-
58	-	-	-	9	58	49	-
59	214	42	193	3145	-	-	-
60	17	319	36	-	-	-	-
61	-	242	203	473	-	-	-
62	9	26	56	-	-	-	27
63	-	-	-	-	50	56	54
64	-	-	-	-	105	191	95
65	423	1039	315	65	-	-	-
66	-	-	-	-	44	217	225
68	-	-	-	-	73	52	-
69	-	-	-	-	55	64	-
70	-	-	-	-	201	239	-
71	26	43	29	-	-	-	-
72	-	-	39	109	41	44	162 (S3)
73	7	8	8	196	1162 S1	378 (S2)	1975
74	-	-	-	-	68	57	-
75	82	59	28	77	701 (S1)	491 (S2)	239
77	36	48	38	44	46	49	35
78	4	46	105	152	93	149	650
80	-	-	-	45	-	2465 (S2)	3171
81	39	19	48	107	-	-	-
82	42	57	38	-	-	790	447 (S3)
83	29	58	54	116	-	-	-
84	-	-	-	21	-	52	34
85	-	-	-	6	72	163	31
86	-	-	-	-	44	34	27
87	-	-	-	44	2881 (S1)	1511	-
89	11	57	48	-	-	-	-
90	13	41	35	132	542	-	-
92	-	-	-	-	77	89	-
96	286	275	-	1182	2376	1807	-
97	-	-	-	-	170	1615	106
99	107	67	114	19	75	42	267 (S3)
104	87	112	231	21	1013	1361 (S2)	-
999	187	276	198	267	246	328	303

Key

SD1 = Sampled 18.03.91
SD2 = Sampled 22.04.91
SD3 = Sampled 15.05.91
C = Culled
CL = Clinical mastitis
= Herd average
- = No information

Appendix VI Most Recent Herd Milk Somatic Cell Count Profile before Sampling Visit

Number of Cows (and Percentage of Herd to nearest 0.5%) in each SMRA Cell Count Band

Farm	1	1	1*	2	3	4**	5	6	7
Date of Visit	18.03.91	22.04.91	13.05.91	15.04.91	20.05.91	01.07.91	16.07.91	30.07.91	29.10.91
Number of cows recorded	50	54	46	145	32	43	83	30	114
Milk Somatic Cell Count									
Under 100,000 cells per ml	26 (52.0%)	22 (40.5%)	23 (50.0%)	40 (27.5%)	11 (34.5%)	1 (2.5%)	11 (13.5%)	5 (16.5%)	29 (25.5%)
100,000 to 250,000 cells per ml	10 (20.0%)	11 (20.5%)	8 (17.5%)	38 (26.0%)	7 (22.0%)	15 (35.0%)	25 (30.0%)	15 (50.0%)	36 (31.5%)
251,000 to 400,000 cells per ml	4 (8.0%)	6 (11.0)	1 (2.0%)	17 (11.5%)	3 (9.5%)	11 (25.5%)	12 (14.5%)	3 (10.0%)	20 (17.5%)
401,000 to 600,000 cells per ml	2 (4.0%)	3 (5.5%)	2 (4.5%)	15 (10.5%)	2 (6.0%)	4 (9.5%)	9 (11.0%)	6 (20.0%)	11 (9.5%)
601,000 to 1 million cells per ml	2 (4.0%)	3 (5.5%)	7 (15.0%)	13 (9.0%)	5 (15.5%)	7 (16.5%)	8 (9.5%)	1 (3.5%)	12 (10.5%)
Over 1 million cells per ml	6 (12.0%)	9 (16.5%)	5 (11.0%)	22 (15.0%)	3 (9.5%)	5 (11.5%)	18 (21.5%)	0 (0%)	6 (5.5%)
Sour sample					1 (3.0%)				
Under 250,000 cells per ml	36 (72.0%)	33.(61.0%)	31 (67.5%)	78 (54.0%)	18 (56.5%)	16 (37.0%)	36 (43.5%)	20 (66.5%)	65 (57.0%)
Over 250,000 cells per ml	14 (28.0%)	21 (39.0%)	15 (32.5%)	67 (46.0%)	13 (40.5%)	27 (63.0%)	47 (56.5%)	10 (33.5%)	49 (43.0%)
Over 600,000 cells per ml	8 (16.0%)	12 (22.0%)	12 (26.0%)	35 (24.0%)	8 (25.0%)	12 (28.0%)	26 (31.5%)	1 (3.5%)	18 (16.0%)
Herd Average Cell Count	246,000	328,000	303,000	453,000	426,000	593,000	523,000	238,000	289,000

* Farm 1 did not continue with the S.M.R.A. scheme after this visit

** Farm 4 did not belong to the S.M.R.A. scheme. Cell counts made on milk samples collected at visit

Results of Isolation Tests on Milk and Other Samples

Cow	MSCC	Source	Blood Agar Staph	Blood Agar Strep	Edwards Agar (aesculin hydrolysis)	Growth in Todd-Hewitt Broth	Inulin Fermentation	API Identification
12	973	LH	-	+	+	-	ND	ND
		LF	-	-	-	ND	ND	ND
		RH	-	+	+	+	+	*A. viridans 2*
		RF	+	+	+	+	+	*A. viridans 3*
		V		ND	+	-	ND	ND
		R		ND	+	+	+	*A. viridans 3*
					+	-	-	ND
16	1530	LH	+	+	-	ND	ND	ND
		LF	-	-	-	ND	ND	ND
		RH	+	+	-	-	-	ND
		RF	+	-	-	ND	ND	ND
		V		ND	+	+	-	*Listeria*
		R		ND	+	+	+	*A. viridans 2/Listeria*
					+	-	+	NIG
22	316	LH	-	+	+	+	+	*A. viridans 3*
					+	+	-	ND
		LF	-	+	+	+	+	*A. viridans 3*
		RH	+	+	+	-	+	NIG
					+	+	+	*A. viridans 2/Listeria*
		RF	+	+	+	+	+	*A. viridans 2*
					+	+	+	*A. viridans 2/Listeria*
					+	+	-	ND
		V		ND	+	-	ND	ND
		R		ND	+	-	ND	ND
34	335	LH		C	+	+	+	*A. viridans 2*
		LF		C	+	+	+	*A. viridans 2/Listeria*
					+	+	+	*A. viridans 2/Listeria*
		RH	+	+	-	ND	ND	ND
		RF	-	-	-	ND	ND	ND
		V		ND	-	ND	ND	ND
		R		ND	-	+	+	*A. viridans 3*
					-	+	-	ND
47	1126	LH	-	-	-	ND	ND	ND
		LF	-	-	-	ND	ND	ND
		RH	-	-	-	ND	ND	ND
		RF	-	-	-	ND	ND	ND
		V		ND	-	ND	ND	ND
		R		ND	-	ND	ND	ND
73	1162	LH	-	-	-	ND	ND	ND
		LF	+	+	+	+	+	*A. viridans 2*
					+	+	+	*A. viridans 3*
					+	+	-	*A. viridans 3*
		RH	-	-	-	-	-	ND
		RF	+	-	-	ND	ND	ND
		V		ND	+	-	-	ND
		R		ND	+	-	+	ND
					+	-	-	ND
75	701	LH	-	+	-	ND	ND	ND
		LF	-	+	+	+	+	*A. viridans 3*
					+	+	+	*A. viridans 2/3*
					+	-	+	ND
		RH	-	+	+	+	+	*A. viridans 2/3*
					+	-	ND	ND
		RF	-	+	-	ND	ND	ND
		V		ND	+	+	+	NIG
					+	-	+	ND
		R		ND	+	+	+	*A. viridans 2/Listeria*
87	2881	LH		C	+	ND	ND	ND
		LF	-	-	-	ND	ND	ND
		RH	-	+	+	ND	ND	ND
		RF	-	-	-	ND	ND	ND
		V		ND	+	ND	ND	ND
		R			+	ND	ND	ND
104	1013	LH		C	+	ND	ND	ND
		LF		C	+	ND	ND	ND
		RH	-	+	+	ND	ND	ND
		RF	-	+	+	ND	ND	ND
		V		ND	+	ND	ND	ND
		R		ND	+	ND	ND	ND

Key

MSCC = Milk somatic cell count (x 1,000 cells per ml)
Staph = Staphylococci
Strep = Streptococci
C = Contamination
\+ = Growth
\- = No growth
ND = Not done
NIG = No identification given

Appendix VIII Farm 1 Visit 2 22.04.92

Results of Isolation Tests on Milk Samples

Cow	MSCC 26.02.91	MSCC 22.03.91	Quarter	Staph	Strep	Edwards Agar	API Identification
5	-	383	LH	+	+	-	ND
			LF	+	+	+(+)	*A. viridans*
			RH	+	-	ND	ND
			RF	+	-	ND	ND
8	293	436	LH	+	-	ND	ND
			LF	-	-	ND	ND
			RH	+	-	ND	ND
			RF	-	-	ND	ND
10	183	405	LH	++	-	ND	ND
			LF	++	-	ND	ND
			RH	++	-	ND	ND
			RF	-	-	ND	ND
12	973	2542	LH	+++	+	+(+)	*A. viridans*
			LF	-	+	+(-)	ND
			RH	+++	-	ND	ND
			RF	++	-	ND	ND
14	218	2705	LH	-	+	-	ND
			LF	+	-	ND	ND
			RH	++	-	ND	ND
			RF	-	-	ND	ND
16	1530	1087	LH	+	+	+(+)	*A. viridans*
			LF	++	-	ND	ND
			RH	+++	-	ND	ND
			RF	Contaminated		ND	ND
18	51	670	LH	-	-	ND	ND
			LF	-	-	ND	ND
			RH	-	-	ND	ND
			RF	-	-	ND	ND
22	316	284	LH	-	-	ND	ND
			LF	+	-	ND	ND
			RH	-	+	-	ND
			RF	-	-	ND	ND
34	335	844	LH	-	-	ND	ND
			LF	+	-	ND	ND
			RH	++	-	ND	ND
			RF	+	+	-	ND
47	1126	1754	LH	+	-	ND	ND
			LF	+	-	ND	ND
			RH	+	+	-	ND
			RF	++	+	+(-)	ND
52	185	253	LH	++	-	ND	ND
			LF	++	-	ND	ND
			RH	+	-	ND	ND
			RF	-	-	ND	ND
53	85	390	LH	-	-	ND	ND
			LF	+	-	ND	ND
			RH	-	-	ND	ND
73	1162	378	LH	+	-	ND	ND
			LF	+	-	ND	ND
			RH	-	-	ND	ND
			RF	-	-	ND	ND
75	701	491	LH	+	-	ND	ND
			LF	+	-	ND	ND
			RH	+	-	ND	ND
			RF	+	-	ND	ND
80	-	2465	LH	+	+	+(+)	*A. viridans*
			LF	+	-	ND	ND
			RH	+	-	ND	ND
			RF	+	-	ND	ND

Key

MSCC = Milk somatic cell count (x 1,000 cells per ml)
Staph = Staphylococci
Strep = Streptococci
LH = Left hind
LF = Left Fore
RH = Right hind
RF = Right fore
\+ = Colony growth
\- = No colony growth
(+) = Aesculin hydrolysis
(-) = No aesculin hydrolysis
ND = Not done

Appendix IX Farm 1 Visit 2 22.04.91

Results of Antimicrobial Susceptibility Tests (Oxoid) on Representative Staphylococcal Colonies from Milk Samples

Cow & Quarter	Ampicillin	Chloramphenicol	Lincomycin	Oxytetracycline	Penicillin G	Streptomycin	Cotrimoxazole
	PN 10µg	C 10µg	MY 2µg	OT 30µg	G 10units	S 10µg	SXT 25µg
10 LH	S	SS	SS	SS	R	SS	SS
12 RH	SS	SS	SS	SS	SS	S	SS
16 RH	SS	SS	SS	S	SS	R	R
34 RH	R	SS	SS	SS	R	SS	SS
73 LF	R	SS	SS	SS	R	SS	SS

Key

SS = Area of susceptibility >1.5cm diameter
S = Area of susceptibility <1.5cm diameter
R = No susceptibility

LH = Left hind
LF = Left fore
RH = Right hind

Cotrimoxazole = Sulphamethazole + Trimethoprim

Appendix X Farm 1 Visit 4 29.08.91

Results of Isolation Tests on Milk Samples

Cow	Inulin Agar 5ml THTP	Inulin Agar 10ml THTP	Blood Agar	Edwards Agar	Hippurate Broth	API Identification
1	-	-	ND	ND	ND	ND
3	-	Y 50	l	s(-)	-	ND
			l	s(-)	-	ND
5A	Y 40	Y 80	l	l(+)	-	ND
			s	pp(+)	-	ND
5B	Y 60	Y 60	s	pp(+)	-	ND
			lm	pp(-)	ND	ND
6	-	-	ND	ND	ND	ND
7	-	Y 70	s	-	-	ND
8	-	Y 5	ND	ND	ND	ND
9	-	Y 60	lmh	pp(-)	ND	ND
			lm	s(-)	ND	ND
11	-	-	ND	ND	ND	ND
12	-	-	ND	ND	ND	ND
13	-	-	ND	ND	ND	ND
14	-	Y 50	ND	ND	ND	ND
15	Y 20	Y 50	-	-	ND	ND
			-	-	ND	ND
16	Y 70	Y 50	lmh	-	ND	ND
			-	-	ND	ND
17	Y 10	Y 50	ND	ND	ND	ND
18	-	-	ND	ND	ND	ND
20	-	-	ND	ND	ND	ND
21	Y 90	Y 80	lm	-	ND	ND
22	Y100	-	h	-	ND	ND
23	-	-	ND	ND	ND	ND
24	-	Y 60	lm	pp(-)	ND	ND
			lm	pp(-)	-	ND
25	-	-	ND	ND	ND	ND
28	Y 40	Y 70	sh	pp(+)	-	ND
			lm	s(-)	ND	ND
30	Y 50	Y 90	s	pp(+)	+	*Str.uberis*
			lm	-	ND	ND
31	-	-	ND	ND	ND	ND
32	Y100	-	lm	-	ND	ND
			-	-	ND	ND
34	-	Y 50	ND	ND	ND	ND
36	-	Y 50	lm	s(+)	-	ND
37	-	-	ND	ND	ND	ND
38	-	Y 40	lm	s(+)	ND	ND
40	-	-	ND	ND	ND	ND
41	-	-	ND	ND	ND	ND
43	-	-	ND	ND	ND	ND
44	-	Y 90	lm	-	ND	ND
			s	pp(+)	-	ND
45	-	Y 80	s	pp(+)	+	*Str.uberis*
			lm	-	ND	ND
46	Y 60	Y 60	lm	-	ND	ND
			s	pp(+)	-	ND
47	-	-	ND	ND	ND	ND
50	-	-	ND	ND	ND	ND
51	-	Y 10	ND	ND	ND	ND
52	Y 90	Y 80	lm	pp(+)	+	NIG
			lm	-	ND	ND
54	Y 40	-	sh	pp(+)	-	ND
			sh	pp(+)	-	ND
55	-	-	ND	ND	ND	ND
56	-	-	ND	ND	ND	ND
58	-	-	ND	ND	ND	ND
60	-	-	ND	ND	ND	ND
62	Y 70	Y 60	s	s(+)	-	*Str.bovis*
			s	s(+)	+	NIG
64	Y 90	Y 80	ND	ND	ND	ND
66	Y 80	Y 70	-	-	ND	ND
67	-	-	ND	ND	ND	ND
68	Y 90	Y 90	lm	-	ND	ND
			lm	pp(+)	ND	ND
69	-	-	ND	ND	ND	ND
70	-	-	ND	ND	ND	ND
71	Y 80	Y100	lm	-	ND	ND
72	-	Y 40	lh	pp(+)	ND	ND
			-	-	ND	ND
73	-	Y 10	ND	ND	ND	ND
74	Y 40	Y 70	lm	pp(+)	ND	ND
75	-	Y 40	ND	ND	ND	ND
77	Y 70	Y 80	lm	-	ND	ND
			-	-	ND	ND
78	-	-	ND	ND	ND	ND
80	-	Y 90	s	s(+)	-	*E.avium*
			-	-	ND	ND
84	-	-	ND	ND	ND	ND
85	-	-	ND	ND	ND	ND
86	-	Y 90	lm	-	ND	ND
89	-	-	ND	ND	ND	ND
92	-	-	ND	ND	ND	ND
99	-	-	ND	ND	ND	ND
104	Y 50	-	s	pp(+)	-	ND

Key

l = Large colony (>2mm diameter)
s = Small colony (<1mm diameter)
pp = Pin-point colony
m = Mucoid colony
Y 20, Y 40 etc = 20%,40% etc agar coloured yellow
h = Beta haemolysis
- = No growth
(+) = Aesculin hydrolysis
(-) = No aesculin hydrolysis
ND = Not done
NIG = No identification given

Appendix XI Farm 1 Visit 5 11.09.91

Results of Isolation Tests on Milk Samples

Cow	Inulin Agar	Edwards Agar	Blood Agar	Hippurate Broth	API Identification
1	Y 75	+(-)	ND	ND	ND
		-	ND	ND	ND
2	Y 75	-	ND	ND	ND
3	Y 75	-	ND	ND	ND
5	Y 0	ND	ND	ND	ND
6	Y 0	ND	ND	ND	ND
7	Y 0	ND	ND	ND	ND
8	Y 0	+(-)	h	ND	ND
9	Y 0	+(-)	mh	ND	ND
11	Y 0	ND	ND	ND	ND
	Y 75	+(+)	mh	ND	ND
		+(-)	ND	ND	ND
12	Y 75	+(+)	mh	ND	ND
		-	ND	ND	ND
13	Y 100	+(+)	lh	ND	ND
		-	ND	ND	ND
14	Y 100	+(+)	lh	ND	ND
		+(-)	ND	ND	ND
		+(+)	sh	ND	ND
15	Y 75	-	ND	ND	ND
16	Y 0	ND	ND	ND	ND
17	Y 0	ND	ND	ND	ND
18	Y 0	ND	ND	ND	ND
20	Y 100	+(+)	lh	ND	ND
		+(+)	mh	ND	ND
21	Y 0	ND	ND	ND	ND
22	Y 100	+(-)	ND	ND	ND
23	Y 0	ND	ND	ND	ND
24	Y 0	ND	ND	ND	ND
25	C	ND	ND	ND	ND

Cow	Inulin Agar	Edwards Agar	Blood Agar	Hippurate Broth	API Identification
28	Y 0	ND	ND	ND	ND
30	Y 0	ND	ND	ND	ND
30LH	-	ND	ND	ND	ND
30LF	Y 0	ND	ND	ND	ND
30RH	-	ND	ND	ND	ND
30RF	-	ND	ND	ND	ND
31	Y 0	ND	ND	ND	ND
32	Y 0	ND	ND	ND	ND
33	Y 0	ND	ND	ND	ND
34	Y 75	+(+)	h	ND	ND
		+(+)	lm	ND	ND
35	Y 75	+(+)	v	+	*S. uberis*
		-	ND	ND	ND
		+(+)	h	ND	ND
36	Y 100	+(+)	h	ND	ND
		-	ND	ND	ND
37	Y 0	ND	ND	ND	ND
38	Y 0	ND	ND	ND	ND
40	Y 50	+(-)	ND	ND	ND
42	Y 0	ND	ND	ND	ND
43	Y 0	ND	ND	ND	ND
44	Y 0	ND	ND	ND	ND
45	Y 100	+(+)	h	ND	ND
45LH	Y 0	+(-)	ND	ND	ND
45LF	-	ND	ND	ND	ND
45RH	Y 0	+(-)	ND	ND	ND
45RF	-	ND	ND	ND	ND
46	Y 0	ND	ND	ND	ND
47	Y 0	ND	ND	ND	ND
50	Y 0	ND	ND	ND	ND

Cow	Inulin Agar	Edwards Agar	Blood Agar	Hippurate Broth	API Identification
51	Y 0	ND	ND	ND	ND
52	Y 0	ND	ND	ND	ND
54	C	ND	ND	ND	ND
56	Y 100	-	ND	ND	ND
58	Y 0	ND	ND	ND	ND
60	Y 0	ND	ND	ND	ND
62	Y 100	+(-)	ND	ND	ND
64	Y 0	ND	ND	ND	ND
66	Y 100	-	ND	ND	ND
67	Y 75	+(-)	ND	ND	ND
68	Y 0	ND	ND	ND	ND
69	Y 100	+(+)	ND	ND	ND
70	Y 0	ND	lm	ND	ND
71	Y 0	ND	ND	ND	ND
72	Y 100	-	ND	ND	ND
73	Y 0	ND	ND	ND	ND
74	C	ND	ND	ND	ND
75	Y 100	+(-)	ND	ND	ND
77	C	ND	ND	ND	ND
78	Y 75	+(-)	ND	ND	ND
		+(+)	lm	ND	ND
80	Y 75	+(-)	ND	ND	ND
82	Y 100	-	ND	ND	ND
84	Y 100	+(-)	ND	ND	ND
85	Y 0	ND	ND	ND	ND
86	Y 0	ND	ND	ND	ND
89	Y 0	ND	ND	ND	ND
92	Y 0	ND	ND	ND	ND
99	Y 100	-	ND	ND	ND
104	Y 0	ND	ND	ND	ND

Key

LH = Left hind
LF = Left fore
RH = Right hind
RF = Right fore

l = Colonies >2mm diameter
s = Colonies <1mm diameter
m = Mucoid colonies
h = Beta haemolysis
v = Viridan haemolysis

\+ = Colony growth
\- = No colony growth
(+) = Aesculin hydrolysis
(-) = No aesculin hydrolysis

Y 50, Y 75 etc = colony growth with 50%, 75% etc agar coloured yellow
Y 0 = colony growth but no agar coloured yellow
ND = Not done
C = Contaminated sample

Appendix XII Farm 2 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml)

Cow No.	Cow Name	01.08.90	26.09.90	25.10.90	21.11.90	19.12.90	25.01.91	01.03.91	04.04.91
1	Elite	D	D	D	D	D	D	1773	2365
2	Madeleine	D	D	D	D	D	3195	2346	2369
3	Bricabrack	D	D	D	D	D	D	D	-
4	Verity	120	224	519	421	597	1383	929	D
5	Celandine	720	1164	S	1530	D	D	D	-
6	Svetlana	-	-	181	416	67	141	163	-
7	Magnolia	1558	6	1558	818	D	D	D	-
8	O.Rosebud	2925	1421	253	D	D	D	D	-
9	H.Candida	D	D	D	D	D	Suckling	-	-
10	Bryony	D	59	65	123	76	Sold	-	-
10	Kate	-	-	-	-	-	-	61	-
11	Quantas	353	D	D	D	35	70	127	-
12	Irene	D	35	90	61	24	710	791	137
13	Mission	181	D	D	D	S	-	4460	1057
14	Ida	2182	D	D	769	865	1694	1194	-
15	Heather	64	58	D	D	47	39	74	-
16	Erica	D	D	128	77	157	41	213	-
17	Matron	-	55	-	59	-	84	141	647
18	Cuckoo	D	D	D	D	D	Suckling	-	-
21	Solveig	21	82	110	410	266	D	D	-
22	Redstart	-	-	2371	441	A/B	151	261	-
23	Nadine	33	41	172	308	150	D	D	-
24	Legs	351	D	D	D	D	D	D	-
25	Melissa	85	D	D	33	84	51	55	-
26	Jane	-	-	102	450	111	253	256	-
27	Moonstone	390	D	D	D	56	1469	85	-
28	Innocent	D	S	105	88	132	255	382	-
29	Chippy	659	321	393	438	D	D	2610	-
30	Vixen	116	S	108	D	48	36	224	-
31	Hollyhock	211	274	337	437	402	867	D	Sold
32	Tanya	212	S	D	D	D	D	119	-
33	Hester	D	90	124	165	676	554	1041	-
34	Debra	D	D	D	D	215	1696	1977	1414
35	Caberet	219	S	487	526	379	D	D	-
36	Lynx	1872	D	76	217	233	1529	1572	863
37	Omega	-	-	178	144	103	86	63	-
38	Kirsten	55	71	55	1846	300	-	1431	-
39	Ruby	D	D	D	D	D	135	173	-
40	Glitz	194	643	D	D	D	D	833	388
41	Delightful	194	617	338	369	501	546	1091	-
42	Zenda	254	538	404	D	D	198	367	-
43	Harebell	-	S	134	100	130	180	294	-
44	Heather	563	D	D	D	D	60	328	-
45	Vashti	206	286	760	1442	-	-	-	-
46	Bandeau	-	145	101	147	120	-	372	-
47	Snowstorm	25	87	97	241	-	525	D	-
48	Custard	65	572	144	165	D	D	88	-
49	Bouquet	664	D	D	D	D	D	D	-
50	Leonora	D	185	54	58	206	86	66	-
51	Marzipan	-	-	-	-	460	859	131	-
52	Dariole	54	507	367	263	D	D	1365	396
53	Venus	D	D	D	D	D	D	D	-
54	G.Fool	61	109	104	87	88	72	40	-
55	Liselot	72	S	184	98	99	139	128	-
56	Dicy	863	D	D	D	D	D	2614	-
57	Velour	538	D	D	D	D	D	118	-
58	Mistake	D	440	1660	164	148	218	40	-
59	Katya	239	D	D	86	75	182	-	-
60	Calico	S	115	104	44	58	-	72	776
61	Elfreda	D	D	D	186	125	71	72	-
62	Rhylda	S	D	D	D	2763	383	546	-
63	Francis	537	2994	3767	Sold	-	-	-	-
63	Fashion	-	-	-	-	95	261	65	-
64	Debutante	15	S	Sick	Sick	Sold	-	-	-
64	Illusion	-	-	-	-	-	830	Sold	-
65	Bettina	305	900	D	D	D	D	51	-
66	Diamond	318	233	1183	316	404	210	434	-
67	Henrietta	2459	S	2450	D	D	D	-	971
68	Midge	D	454	56	146	66	A/B	1013	-
69	Idaho	184	130	390	-	455	D	D	-
70	Suprise	-	-	76	67	126	139	44	-
71	Moonlight	907	D	D	D	D	D	659	287
72	F.Minuet	939	D	D	D	D	D	211	305
73	D.Pastry	162	99	418	D	D	102	498	289
74	Garnet	27	75	94	107	64	90	176	-
75	Mocha	D	D	D	D	D	-	227	947
76	Miss Ad	388	D	D	43	46	221	105	-

Appendix XII Farm 2 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml) continued

Cow No.	Cow Name	01.08.90	26.09.90	25.10.90	21.11.90	19.12.90	25.01.91	01.03.91	04.04.91
77	Snowfire	136	29	85	45	152	-	45	-
78	Venture	163	213	202	233	499	374	463	-
79	Lily	94	43	61	67	60	57	84	-
80	Concorde	643	125	D	D	D	D	154	-
81	M.Candida	D	D	D	D	D	-	-	-
82	Hosta	-	125	196	206	38	46	-	-
83	Britta	-	89	113	124	219	D	-	-
84	Melody	-	34	65	79	55	-	108	-
85	Butterfly	D	D	264	1468	593	270	409	-
86	Vandal	373	D	D	D	D	D	39	-
87	Canberra	D	169	1258	Sick	Sold	-	-	-
87	Heidi	-	-	-	-	-	-	192	-
88	Minaret	64	201	261	174	D	D	-	-
89	Mrs Churn	509	810	253	D	D	D	-	330
90	Halleluah	D	D	D	D	D	D	-	-
91	Dorrinda	D	91	120	165	389	169	-	-
92	Comet	184	D	D	D	D	D	D	698
93	Anthem	330	467	S	D	D	85	1261	-
94	Dumpling	1325	1138	1048	D	D	D	-	-
95	Mistletoe	-	48	-	38	71	63	36	-
96	Saucy	-	52	S	79	55	97	72	2433
97	Jessica	45	289	101	206	434	1157	860	D
98	Mishap	3891	D	D	D	D	D	716	1695
100	Twinkle	2355	1020	2326	2630	Suckling	Sold	-	-
101	Rhythm	-	S	-	99	30	83	22	-
102	Thumbelina	-	264	613	609	Sold	-	-	-
104	Renown	131	186	896	Sold	-	-	-	-
105	Tivoli	524	345	757	1330	1671	-	1514	-
106	G.Gracious	176	D	D	128	113	66	202	-
107	Greylag	179	D	D	143	55	96	21	-
108	C.Bun	D	D	D	D	D	-	Sold	-
109	D.Delight	-	-	148	71	65	69	33	-
110	Jura	238	S	238	D	D	57	38	-
111	Gina	-	25	192	35	41	28	78	-
112	Gavotte	690	D	D	D	D	D	-	-
113	Chloe	D	D	D	D	D	264	128	2102
114	Destiny	164	D	D	102	337	-	425	275
115	Merry	-	S	227	Sick	Sick	Suckling	614	-
116	Damsel	6380	986	D	D	D	-	-	-
117	Trudy	2822	S	545	692	D	D	337	476
118	Renate	263	D	D	D	D	-	109	371
119	Ragbag	D	D	D	53	-	-	554	Sold
120	Mona	5871	S	Sold	-	-	-	-	-
120	Monarda	-	-	-	-	-	48	55	-
121	Lightning	-	S	175	97	215	144	189	-
122	Rasmine	161	95	S	133	99	79	138	-
123	Michaelmas	-	-	124	79	46	55	551	-
124	Crumpet	129	S	284	195	1835	-	311	-
125	Myrna	-	-	1584	A/B	264	-	718	-
126	Eileen	D	D	D	D	D	D	-	-
127	Magda	39	38	72	2364	204	287	78	-
128	Kookaburra	D	D	D	D	D	D	D	-
129	Rebecca	-	-	-	222	222	284	124	-
130	Parsley	75	1423	1076	D	D	-	-	-
131	Spitfire	153	198	193	121	2116	118	D	-
132	Lady	1656	5346	3705	D	D	2385	437	3900
133	Return	1176	532	1552	670	777	945	1289	726
134	Ladybird	938	1033	884	319	802	839	Sold	-
135	Flashy	-	-	-	73	43	44	46	-
136	Melanie	44	213	233	314	327	1064	D	-
137	R.Clover	-	-	-	90	298	204	429	-
138	Imogen	-	-	-	86	816	177	121	-
139	Doughnut	3691	2038	2492	1052	Suckling	-	-	-
140	J.Svena	312	-	596	203	54	42	70	-
142	Kingfisher	731	2115	1200	D	D	182	2204	2863
144	Bracken	168	A/B	645	194	236	1255	-	-
145	Poppy	D	203	625	344	656	402	1811	-
146	Razzmatazz	662	D	D	515	242	-	326	-
147	Starlight	247	339	461	351	416	284	253	-
149	Myrtle	45	D	50	75	478	1003	1240	-
150	Bunty	D	D	D	D	128	165	-	-
151	Bumble B	311	D	D	D	D	D	920	-
152	Violet	D	639	361	74	53	-	107	-
154	Nougat	-	-	-	63	54	40	58	-
156	Mercury	D	D	D	D	164	63	314	-
157	Jean	D	38	60	1018	127	-	179	-
158	Deutzia	171	537	51	248	164	97	D	-

Appendix XII Farm 2 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml) concluded

Cow No.	Cow Name	01.08.90	26.09.90	25.10.90	21.11.90	19.12.90	25.01.91	01.03.91	04.04.91
159	Gerry	633	275	323	1488	D	D	-	-
160	Quickstep	120	158	205	240	239	1453	495	-
161	Midnight	351	171	627	302	475	-	D	-
162	Harvest	D	85	48	49	71	226	695	-
163	Birch 4	D	D	D	D	D	D	D	-
167	Charlston	56	68	157	100	168	137	D	-
168	M. Mallow	922	S	673	385	D	D	D	-
169	F. Svena	22	300	148	168	217	353	D	-
170	Mango	366	D	D	D	D	D	D	-
171	Evita	296	D	D	D	D	D	100	-
172	Nightshade	104	574	491	D	D	D	D	-
174	Capsium	191	26	D	D	D	26	43	-
175	Teazel	228	162	247	244	1444	390	D	-
177	Genevive	411	D	D	2289	2203	2049	2246	-
178	Auricula	D	D	D	D	119	132	Sold	-
179	Birch 8	223	D	53	100	93	218	240	-
180	Romany	450	D	59	405	402	635	908	-
181	Daisy	194	98	188	112	213	D	122	-
186	Junker	64	S	D	D	D	66	103	-
189	May	1724	D	D	D	D	D	1764	1158
190	Silver	178	279	471	D	D	71	145	-
194	Marion	94	57	95	1753	647	162	D	-
195	Coleen	220	340	644	514	D	D	D	-
197	V. Minnuet	D	76	53	82	36	-	-	-
198	Fourleaf	2826	S	103	D	D	242	95	242
200	D. Deluge	351	S	S	91	172	471	D	-
202	Rosebay	22	29	55	69	109	179	84	-
203	Coronelle	1	S	81	95	111	375	236	-
204	Caravelle	204	1033	331	357	374	911	379	-
205	Verona	62	128	187	98	126	215	276	-
207	Chance	1091	468	971	1856	D	D	D	-
208	Tessa	28	33	51	117	73	92	86	-
209	Rialto	139	S	A/B	2623	141	208	478	-
210	Zulu	99	66	240	250	163	306	436	-
213	B.Princess	D	S	107	524	260	217	251	-
214	D.Princess	115	S	A/B	705	304	424	D	-
216	Helege	10	S	80	98	57	-	221	-
217	Inge	D	D	D	D	573	Suckling	-	-
219	D. Svena	D	D	D	36	63	-	397	279
220	Sally	D	D	D	D	D	D	58	-
221	Crusty	D	D	D	D	Sold	-	-	-
222	V. Deluge	197	D	D	D	D	D	D	-
223	Snowdrop	269	D	D	D	D	D	104	-
224	Niobe	D	S	72	45	72	Sold	-	-
225	Contrast	D	D	D	D	161	132	510	-
226	Blackberry	366	S	D	D	D	D	D	-
227	Legend	D	D	D	475	212	191	168	-
228	Ruler	D	D	D	D	1054	352	Sold	-
229	Hopeful	D	S	53	2562	63	-	129	-
230	Kay	D	D	D	D	D	D	D	-
232	Hickory	110	180	509	211	253	D	651	65
233	Heda	27	S	S	D	D	47	71	-
234	Kedron	1335	844	444	718	573	D	912	2773
235	Laughter	D	37	284	216	324	239	587	-
236	Velcro	169	394	278	400	215	210	D	-
237	Sunflower	675	D	D	D	D	148	256	-
238	Muddle	640	D	D	D	D	D	D	-
239	Melba	D	D	D	58	47	46	67	-
240	Lynette	104	D	D	D	D	D	D	-
241	Irmgard	298	D	57	84	214	D	D	-
242	Rowena	D	D	87	48	61	-	156	-
243	Kylin	212	D	D	D	D	-	3206	3292
244	Croissant	D	D	S	69	66	105	Sold	-
245	Vagrant	D	D	D	D	D	D	-	-
246	Casino	1682	D	D	D	D	-	53	-
247	Elvera	S	D	D	D	D	D	56	-
248	Rhoda	438	D	D	D	D	D	39	-
250	Delta	D	D	D	D	D	D	-	-
251	Apple	482	157	210	208	172	-	-	-
252	Lyrebird	D	D	D	208	1072	1048	-	1327
253	Kalinka	125	D	D	D	D	D	-	-
254	Wonderous	21	D	D	D	40	36	68	-
255	Jalna	38	S	D	D	D	D	D	-
257	Topaz	56	102	S	134	D	-	226	339

Key D = Dry A/B = Antibiotic Treatment
S = Sour Sample - = No Information Available

Appendix XIII Farm 2 Visit 1 15.04.91 Results of Isolation Tests on Milk Samples

Cow	Housing	Somatic Cell Counts (x 1,000 cells per ml) 01.03.91	04.04.91	Quarter	Blood Agar Staph	Edwards Agar Strep	API Identification
2	straw yard	2346	2369	LH	+	m (+)	ND
				LF	+	m (+)	ND
				RH	+	l (+)	ND
				RF	+	l (+)	ND
17	cubicles	?	?	LH	-	-	ND
				LF	-	-	ND
				RH	+	s (+)	*A.viridans*
				RF	-	-	ND
28	cubicles	382	?	LH	+	-	ND
				LF	-	-	ND
				RH	+	m (+)	ND
				RF	+	m (+)	ND
34	cubicles	1696	?	LH	+	m (+)	ND
				LF	c	m (+)	ND
				RH	+	m (+)	ND
				RF	+	m (+)	ND
36	straw yard	1572	863	LH	+	l (+)	ND
				LF	-	-	ND
				RH	+	-	ND
				RF	+	-	ND
40	cubicles	833	388	LH	+	-	ND
				LF	+	-	ND
				RH	+	m (+)	ND
				RF	+	m (+)	ND
52	cubicles	1365	396	LH	+	m (-)	ND
				LF	+	-	ND
				RH	+	m (-)	ND
				RF	c	-	ND
56	straw yard	2614	186	LH	+	m (+)	ND
				LF	+	m (+)	ND
				RH	-	-	ND
				RF	+	-	ND
67	cubicles	?	971	LH	+	l (+)	ND
				LF	+	l (+)	ND
				RH	+	l (+)	ND
				RF	+	l (+)	ND
						s (-)	ND
72	cubicles	211	305	LH	+	s (-)	ND
				LF	+	l (+)	ND
				RH	c	s (-)	ND
				RF	+	-	ND

Appendix XIII Farm 2 Visit 1 15.04.91 Results of Isolation Tests on Milk Samples (concluded)

Cow	Housing	Somatic Cell Counts (x 1,000 cells per ml) 01.03.91	04.04.91	Quarter	Blood Agar Staph	Edwards Agar Strep	API Identification
75	straw yard	227	947	LH	+	l (-)	ND
						s (-)	ND
				LF	+	m (-)	ND
				RH	+	m (+)	ND
				RF	+	m (+)	ND
92	straw yard	?	?	LH	+	m (+)	ND
				LF	+	-	ND
				RH	+	m (+)	ND
				RF	+	-	ND
96	cubicles	72	2433	LH	+	s (+)	*A.viridans*
				LF	+	-	ND
				RH	+	-	ND
				RF	+	-	ND
98	straw yard	716	1695	LH	+	m (+)	ND
				LF	+	m (+)	ND
				RH	+	-	ND
				RF	-	-	ND
132	straw yard	437	3900	LH	+	m (+)	ND
				LF	+	m (+)	ND
				RH	+	m (+)	ND
				RF	+	s (+)	*A.viridans*
142	cubicles	2204	2863	LH	+	l (+)	ND
				LF	-	-	ND
				RH	+	m (+)	ND
				RF	+	-	ND
189	straw yard	1764	1158	LH	+	-	ND
				LF	+	m (+)	ND
				RH	+	s (-)	ND
				RF	+	m (+)	
232	cubicles	651	65	LH	+	s (-)	ND
				LF	-	-	ND
				RH	+	m (+)	ND
				RF	+	m (+)	ND
234	cubicles	912	2773	LH	+	m (+)	ND
						s (-)	ND
				LF	+	m (+)	ND
				RH	+	m (+)	ND
				RF	+	m (+)	
252	cubicles	?	1327	LH	+	m (+)	ND
				LF	-	-	ND
				RH	+	s (+)	*A.viridans*

Key

+ = Growth
- = No gowth
l = Colonies >2mm diameter (24 hours)
m = Colonies 1 - 2 mm diameter (24 hours)
s = Colonies <1mm diameter (24 hours)

(+) = Aesculin hydrolysis
(-) = No aesculin hydrolysis
c = Contamination
? = No information available
ND = Not defined

Appendix XIV Farm 2 Visit 2 01.05.91

Results of Antimicrobial Susceptibility Tests (Oxoid) on Representative Staphylococcal Colonies from Milk Samples

Cow & Quarter	Ampicillin PN 10µg	Chloramphenicol C 10µg	Lincomycin MY 2µg	Oxytetracycline OT 30µg	Penicillin G G 10units	Streptomycin S 10µg	Cotrimoxazole SXT 25µg	Amoxycillin AML 25µg	Amoxycillin + Clavulanic Acid AMC 30µg	Cloxacillin OB 5µg	Cefuroxime CXM 5µg	Erythromycin E 10µg	Neomycin N 10µg	Novobiocin NV 5µg
17 RH	SS	SS	SS	R	S	R	SS	R	SS	SS	R	SS	S	SS
28 RH	R	SS	SS	SS	R	S	SS	R	SS	SS	R	SS	S	S
34 LH	R	SS	SS	SS	R	S	SS	S	SS	SS	S	SS	S	SS
34 RH	R	SS	SS	SS	R	S	SS	R	SS	SS	R	SS	S	S
67 LH	R	SS	S	SS	R	S	SS	R	SS	SS	R	SS	S	S
67 LF	R	SS	SS	R	R	R	SS	R	SS	S	S	R	R	S
72 LH	R	SS	SS	SS	R	S	SS	S	S	S	R	SS	SS	S
72 LF	SS	SS	SS	SS	SS	S	SS	SS	SS	SS	SS	SS	S	SS
117 LH	R	SS	SS	SS	R	S	SS	R	SS	SS	SS	SS	S	SS
117 RH	SS	SS	R	R	SS	SS	R	S	SS	R	R	S	S	R
232 LH	R	SS	SS	R	R	R	SS	S	SS	SS	SS	SS	S	SS
252 LH	R	SS	SS	SS	R	S	SS	R	SS	SS	SS	SS	S	SS
252 RH	R	SS	R	SS	R	S	SS	R	SS	SS	SS	SS	S	SS

Key

SS = Area of susceptibility > 1.5cm diameter
S = Area of susceptibility < 1.5cm diameter
R = No susceptibility

LH = Left hind
LF = Left fore
RH = Right hind

Cotrimoxazole = Sulphamethazole + Trimethoprim

Appendix XV Farm 3 Visit 23.05.91

SMRA Milk Somatic Cell Counts (x 1,000 cells per ml)

Cow Number	08.05.91	
1	0	S
3	56	
4	27	
5	127	
6	2383	
7	97	
10	606	SD
11	511	SD
12	180	
13	161	
15	328	SD
16	117	
17	708	SD
18	384	SD
19	802	SD
20	93	
21	133	
22	242	
23	83	
25	1097	SD
27	74	
28	46	
34	53	
35	69	
36	44	
39	421	SD
44	744	SD
45	193	
46	52	
47	372	
49	858	SD
50	2160	
999	426	

Key
S = Sour Sample
SD = Sampled 20.05.91
999 = Herd Average

Appendix XVI Farm 3 Visit 23.05.91

Results of Isolation Tests on Milk Samples

Cow	MSCC	Age (years)	Date of Calving	Quarter	Blood Agar Staph	Strep	Edwards Agar	Inulin Broth
10	606	9	17.03.91	LH	+	-	-	ND
				RH	+	-	-	ND
				RF	+	-	-	ND
11	511	8	26.02.91	LH	-	-	-	ND
				LF	+	-	-	ND
				RH	++	-	+(-)	ND
				RF	-	-	-	ND
15	328	8	12.10 90	LH	+	-	-	ND
				LF	+	-	-	ND
				RF	+	-	-	ND
17	708	9	14.02.91	LH	+	-	+(-)	ND
				LF	++	-	+(-)	ND
				RH	+++	-	+(+)	-
				RF	+	-	+(+)	-
18	364	9	02.04.91	LH	+	-	+(+)	-
				LF	+	-	+(-)	ND
				RH	+	-	+(-)	ND
				RF	+	-	-	ND
19	802	8	11.12.91	LH	+	-	-	ND
				LF	+	+	+(+)	-
				RH	+	-	-	ND
				RF	++	-	+(-)	ND
25	1097	8	26.02.91	LH	+++	-	+(-)	ND
				RH	++	-	-	ND
				RF	++	-	+(-)	ND
39	421	8	20.03.91	LH	-	-	-	ND
				LF	-	-	-	ND
				RH	+	-	-	ND
				RF	++	-	+(-)	ND
44	744	7	16.02.91	LH	+	-	-	ND
				RH	++	-	+(-)	ND
49	858	8	22.06.90	LH	+	+	+(-)	ND
				LF	+	-	-	ND
				RH	+	-	-	ND
				RF	+	-	-	ND

Key MSCC = Milk somatic cell count (x 1,000 cells per ml)
+ = Colony growth
- = No growth
+(+) = Colony growth with aesculin hydrolysis
+(-) = Colony growth with no aesculin hydrolysis
ND = Not done

Appendix XVII Farm 3 Visit 23.05.91

Results of Antimicrobial Susceptibility Tests (Oxoid) on Representative Staphylococcal Colonies from Milk Samples

(Two colonies taken from one milk sample)

Cow & Quarter	Ampicillin	Chloramphenicol	Lincomycin	Oxytetracycline	Penicillin G	Streptomycin	Cotrimoxazole	Amoxycillin	Amoxycillin + Clavulanic Acid	Cloxacillin	Cefuroxime	Erythromycin	Neomycin	Novobiocin
	PN 10µg	C 10µg	MY 2µg	OT 30µg	G 10units	S 10µg	SXT 25µg	AML 25µg	AMC 30µg	OB 5µg	CXM 5µg	E 10µg	N 10µg	NV 5µg
10 RH	SS	SS	S	SS	SS	S	R	SS	SS	SS	SS	SS	S	R
10 RF	R	SS	S	SS	R	R	SS	R	S	SS	SS	SS	S	SS
11 RH	SS	SS	SS	SS	SS	S	S	SS	SS	SS	SS	SS	S	SS
15 LF	R	SS	S	S	R	S	S	R	SS	SS	SS	SS	S	SS
17 LF	SS	SS	R	SS	SS	R	SS	SS	SS	S	S	SS	S	R
17 RH	SS	SS	S	SS	SS	S	S	SS	SS	SS	SS	SS	S	R
17 RH	S	SS	S	S	R	R	R	S	SS	R	R	SS	R	R
18 LF	SS	SS	S	SS	SS	R	S	SS	SS	SS	SS	SS	S	S
19 LF	SS	SS	S	SS	SS	R	S	SS	SS	SS	SS	SS	S	S
25 RF	SS	SS	S	SS	SS	S	S	SS	SS	SS	SS	SS	S	S
44 RH	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	S	S
49 RF	S	SS	S	SS	S	R	SS	S	SS	S	S	SS	S	R

Key

SS = Area of susceptibility > 1.5cm diameter
S = Area of susceptibility < 1.5cm diameter
R = No susceptibility

LF = Left fore
RH = Right hind
RF = Right fore

Cotrimoxazole = Sulphamethazole + Trimethoprim

Appendix XVIII Farm 4 Visit 01.07.91

Milk Somatic Cell Counts and Results of Isolation Tests on Milk Samples

Cow	Average MSCC	Quarter	Quarter MSCC	Blood Agar Staph	Blood Agar Strep	Edwards Agar	API Test
1	511	LH	397	+	-	ND	ND
		LF	480	+	-	ND	ND
		RH	716	+	-	ND	ND
		RF	449	+	+	-	ND
5	225	LH	239	++	+	-	ND
		LF	173	+	+	-	ND
		RH	338	-	+	-	ND
		RF	150	+	+	-	ND
9	104	LH	114	+++	-	ND	ND
		LF	104	++	-	ND	ND
		RH	84	+++	-	ND	ND
		RF	112	+++	-	ND	ND
10	705	LH	1160	+++	-	ND	ND
		LF	280	+	+	-	ND
		RH	1000	+	+	-	ND
		RF	380	+	+	-	ND
11	186	LH	253	++	-	ND	ND
		LF	202	+	-	ND	ND
		RH	151	+	-	ND	ND
		RF	139	+	+	-	ND
15	1770	LH	396	-	+	+	ND
		LF	2557	++++	+	+	ND
		RH	4043	+	+	+	ND
		RF	83	++	-	ND	ND
17	289	LH	353	+	+	-	ND
		LF	242	-	+	-	ND
		RH	256	-	+	-	ND
		RF	303	++	-	ND	ND
23	279	LH	327	++	+	-	ND
		LF	224	++	-	ND	ND
		RH	247	++	+	-	ND
		RF	318	++	+	-	ND
25	347	LH	410	++	+	-	ND
		LF	263	-	+	-	ND
		RH	270	+	+	-	ND
		RF	443	++	+	-	ND
26	223	LH	306	-	-	ND	ND
		LF	187	-	-	ND	ND
		RH	177	-	-	ND	ND
29	192	LH	160	++	-	ND	ND
		LF	156	+	-	ND	ND
		RH	92	-	-	ND	ND
		RF	360	-	-	ND	ND
30	669	LH	242	-	-	ND	ND
		LF	307	+	-	ND	ND
		RH	492	+	-	ND	ND
		RF	1636	+	-	ND	ND
32	657	LH	317	-	-	ND	ND
		LF	1669	-	+	+	*L. lactis*
		RH	171	-	-	ND	ND
		RF	471	+	-	ND	ND
35	250	LH	221	++	-	ND	ND
		LF	114	+++	-	ND	ND
		RH	302	++	-	ND	ND
		RF	362	-	-	ND	ND
38	1958	LH	346	-	-	ND	ND
		LF	773	-	-	ND	ND
		RH	6271	-	+	+	*L. lactis*
		RF	443	-	-	ND	ND

Appendix XVIII Farm 4 Visit 01.07.91 (continued)

Milk Somatic Cell Counts and Results of Isolation Tests on Milk Samples

Cow	Average MSCC	Quarter	Quarter MSCC	Blood Agar Staph	Blood Agar Strep	Edwards Agar	API Test
43	5005	LH	292	+	-	ND	ND
		LF	246	-	-	ND	ND
		RH	710	-	-	ND	ND
		RF	18772	-	+	+	*L. lactis*
52	135	LH	147	++	-	ND	ND
		LF	100	-	-	ND	ND
		RH	134	+	-	ND	ND
		RF	159	-	+	ND	ND
55	492	LH	440	-	-	ND	ND
		LF	568	-	+	ND	ND
		RH	420	++	+	ND	ND
		RF	540	-	-	ND	ND
58	241	LH	328	+++	-	ND	ND
		LF	216	-	+	ND	ND
		RH	265	++	+	ND	ND
		RF	154	-	+	ND	ND
60	766	LH	563	++	+	-	ND
		LF	551	+	+	+	*L. lactis*
		RH	444	+	+	-	ND
		RF	1505	+	-	ND	ND
61	364**	LH	404	+	+	-	ND
		LF	S	++	-	ND	ND
		RH	323	-	+	-	ND
		RF	S	-	+	-	ND
63	803	LH	250	+	-	ND	ND
		LF	985	+	+	-	ND
		RH	1167	+	-	ND	ND
		RF	811	++	-	ND	ND
69	287	LH	124	-	-	ND	ND
		LF	275	+	+	-	ND
		RH	187	+	+	-	ND
		RF	562	+++	-	ND	ND
71*	186	LH	170	-	-	ND	ND
		LF	102	-	-	ND	ND
		RH	215	-	-	ND	ND
		RF	258	-	-	ND	ND
75	307	LH	374	-	-	ND	ND
		LF	272	-	-	ND	ND
		RH	356	-	-	ND	ND
		RF	224	-	-	ND	ND
76	228	LH	308	+	-	ND	ND
		LF	167	+	-	ND	ND
		RH	282	++	-	ND	ND
		RF	154	++	-	ND	ND
78	401	LH	580	++	-	ND	ND
		LF	143	++	-	ND	ND
		RH	700	+	-	ND	ND
		RF	180	+	-	ND	ND
84	322	LH	291	+	-	ND	ND
		LF	379	-	-	ND	ND
		RH	326	+	-	ND	ND
		RF	292	-	-	ND	ND
92	1783	LH	1105	++	-	ND	ND
		LF	1946	+++	-	ND	ND
		RH	3004	++	-	ND	ND
		RF	1078	+++	-	ND	ND
95	283	LH	199	-	+	-	ND
		LF	164	-	-	ND	ND
		RH	602	+	+	+	*L. lactis*
		RF	168	+	-	ND	ND

Appendix XVIII Farm 4 Visit 01.07.91 (concluded)

Milk Somatic Cell Counts and Results of Isolation Tests on Milk Samples

Cow	Average MSCC	Quarter	Quarter MSCC	Blood Agar Staph	Blood Agar Strep	Edwards Agar	API Test
100	264	LH	471	++	-	ND	ND
		RH	114	-	-	ND	ND
		RF	206	-	-	ND	ND
101	1519	LH	4860	++	-	ND	ND
		LF	628	-	-	ND	ND
		RH	300	-	+	-	ND
		RF	286	+	+	-	ND
102	297	LH	149	-	-	ND	ND
		LF	197	-	-	ND	ND
		RH	291	++	-	ND	ND
		RF	550	-	+	-	ND
108	603	LH	720	++	-	ND	ND
		LF	150	+	+	-	ND
		RH	1100	+	+	-	ND
		RF	440	+	+	-	ND
110	107	LH	81	-	-	ND	ND
		LF	174	++	-	ND	ND
		RH	68	-	+	-	ND
		RF	103	-	-	ND	ND
112	128	LH	160	++	-	ND	ND
		LF	128	-	-	ND	ND
		RH	93	-	-	ND	ND
		RF	129	-	-	ND	ND
114	780	LH	966	-	+	-	ND
		LF	606	-	-	ND	ND
		RH	748	-	-	ND	ND
		RF	798	+	-	ND	ND
117	393	LH	666	+	+	-	ND
		LF	600	+	+	-	ND
		RH	220	-	-	ND	ND
		RF	85	-	+	-	ND
118	181	LH	187	-	-	ND	ND
		RH	196	-	+	-	ND
		RF	159	-	-	ND	ND
120	195	LH	368	-	-	ND	ND
		LF	138	++	-	ND	ND
		RH	98	++	-	ND	ND
		RF	177	++	-	ND	ND
124	425	LH	152	-	+	-	ND
		LF	408	-	+	-	ND
		RH	924	++	-	ND	ND
		RF	216	-	+	-	ND
125	70	LH	73	-	-	ND	ND
		LF	78	-	-	ND	ND
		RH	60	-	-	ND	ND
		RF	70	-	-	ND	ND
126*	202	LH	297	-	-	ND	ND
		LF	216	+	+	-	ND
		RH	107	+	+	-	ND
		RF	187		-	ND	ND

Key
MSCC = Milk somatic cell count (x 1,000 cells per ml)
\+ = Colony growth
(+) = Aesculin hydrolysis
\- = No colony growth or colony growth without aesculin hydrolysis
* = Cow with recent history of S. uberis infection
** = Average MSCC calculated from 2 quarters
ND = Not done
S = Sour sample

Appendix XIX Farm 4 Visit 01.07.91

Results of Antimicrobial Susceptibility Tests (Oxoid) on Representative Staphylococcal Colonies from Milk Samples

(Two colonies taken from some milk samples)

Cow & Quarter	Ampicillin PN 10µg	Chloramphenicol C 10µg	Lincomycin MY 2µg	Oxytetracycline OT 30µg	Penicillin G G 10units	Streptomycin S 10µg	Cotrimoxazole SXT 25µg	Amoxycillin AML 25µg	Amoxycillin + Clavulanic Acid AMC 30µg	Cloxacillin OB 5µg	Cefuroxime CXM 5µg	Erythromycin E 10µg	Neomycin N 10µg	Novobiocin NV 5µg
9 RF	SS	S	S	S	SS	S	S	S	SS	S	S	S	S	S
15 LF	R	SS	S	R	R	SS	S	S	SS	SS	S	SS	S	S
15 LF	SS	SS	SS	SS	SS	R	S	SS	SS	S	S	SS	S	S
23 LH	S	S	S	R	R	R	R	S	SS	SS	SS	SS	S	SS
35 RH	SS	SS	SS	SS	SS	S	S	SS	SS	SS	S	SS	S	S
35 RH	SS	SS	S	S	SS	S	S	SS	SS	S	S	S	S	S
58 LH	SS	S	S	S	SS	S	S	SS	SS	SS	S	S	S	R
58 LH	SS	SS	S	SS	SS	SS	S	SS	SS	SS	SS	SS	S	R
61 LF	R	SS	R	SS	R	S	R	R	S	R	S	R	R	R
61 LF	SS	SS	SS	SS	SS	R	SS	SS	SS	S	SS	SS	S	S
69 RF	SS	SS	SS	SS	SS	SS	R	SS	SS	SS	SS	SS	SS	S
76 RF	SS	SS	SS	S	SS	SS	R	SS	SS	SS	SS	SS	S	SS
76 RF	SS	SS	SS	SS	SS	SS	S	SS	SS	SS	SS	SS	S	S
92 LH	SS	SS	S	SS	SS	S	R	SS	SS	SS	S	S	S	S
126 RH	SS	SS	SS	R	S	S	S	SS	SS	SS	SS	SS	S	R
126 RH	SS	SS	SS	SS	SS	S	S	S	SS	SS	SS	SS	S	S

Key

SS = Area of susceptibility > 1.5cm diameter
S = Area of susceptibility < 1.5cm diameter
R = No susceptibility

LH = Left hind
LF = Left fore

RH = Right hind
RF = Right fore

Cotrimoxazole = Sulphamethazole + Trimethoprim

Appendix XX Farm 5 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml)

Cow No.	06.11.90	11.12.90	15.01.91	12.02.91	08.03.91	11.04.91	11.06.91	06.07.91
2	185	59	17	51	1360	1031	2572	1378
3	1539	2780	-	-	-	-	-	-
4	85	80	60	70	150	173	202	78
5	238	330	-	-	130	87	123	122
6	-	800	60	60	120	108	119	113
7	39	280	2060	1350	3390	1773	1059	883
8	50	80	110	70	100	674	393	-
12	51	60	60	50	60	114	123	65
13	330	420	-	-	-	-	-	-
14	47	1450	300	220	90	107	239	415
15	451	430	S	240	210	719	1348	-
16	211	290	140	390	210	523	-	-
17	214	80	410	3720	920	2350	1386	-
18	181	90	70	770	270	290	165	-
19	170	140	-	-	-	-	-	-
20	55	70	60	120	150	-	-	-
21	233	1240	400	520	2250	5941	1016	-
23	-	240	910	1440	2310	2137	1361	1680
24	1900	190	660	380	880	453	-	68
25	-	-	-	-	-	125	95	52
27	275	1320	350	670	360	1340	791	-
28	103	90	1540	2360	150	263	-	-
30	166	280	150	260	150	137	236	208
31	-	-	-	-	-	-	-	34
32	966	750	160	230	140	993	390	476
33	283	320	250	-	-	-	-	-
34	-	-	-	670	1760	713	1042	903
35	109	510	S	1170	760	745	2928	506
37	529	490	260	610	370	304	599	919
38	51	100	90	80	180	89	107	-
40	5653	-	-	-	-	-	-	-
41	301	80	S	-	350	279	244	116
44	2155	190	180	350	240	724	667	-
45	523	260	480	460	560	1695	-	-
46	97	100	370	90	170	-	463	57
47	100	70	130	-	-	1179	1048	1416
48	166	150	360	460	2700	-	108	165
50	119	80	640	150	220	261	526	205
51	107	860	2010	180	80	91	163	113
52	-	-	80	4730	2010	5669	4104	5235
54	46	70	40	630	870	664	826	1890
55	148	183	260	120	210	1409	2367	-
56	80	40	80	90	88	1107	932	463
57	434	640	960	2830	1070	-	-	-
58	-	-	180	-	-	-	-	-
59	253	480	1830	-	-	-	-	-
61	-	330	60	106	80	245	225	306
62	484	420	370	-	-	1266	217	208
63	-	-	-	-	-	113	149	174
65	100	100	160	350	110	231	406	989
66	-	-	-	-	450	92	98	127
67	545	2130	140	420	1450	968	723	2652
68	1561	-	-	-	-	-	-	-
69	240	300	190	230	2010	3027	878	959
70	1232	290	990	890	710	-	-	-
71	428	610	-	80	440	1139	2283	1322
72	88	190	200	240	240	242	544	-
73	279	190	180	1240	690	550	1690	207
74	57	100	110	110	130	157	653	2359
75	448	850	930	1340	2710	1425	975	-
76	509	190	-	440	450	769	481	352
77	118	120	130	140	160	749	-	226
78	210	230	210	330	180	394	-	432
79	277	120	770	1810	780	448	681	333
80	314	110	130	1320	480	213	885	-
81	-	-	70	50	50	62	111	102
82	-	100	110	160	490	415	128	1562
83	61	-	-	-	-	-	-	-

Appendix XX Farm 5 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml) concluded

Cow No.	06.11.90	11.12.90	15.01.91	12.02.91	08.03.91	11.04.91	11.06.91	06.07.91
84	155	190	130	190	150	136	86	117
85	47	50	110	140	70	1643	205	-
86	133	510	320	350	580	322	-	55
87	240	210	730	470	790	1166	2996	-
88	130	160	180	180	190	278	975	1933
89	71	80	80	110	180	180	184	140
90	-	-	-	-	-	110	218	593
91	105	220	170	230	340	1634	616	1455
94	262	200	270	460	430	676	1124	-
96	82	430	260	190	400	768	1055	-
97	1076	190	740	2230	1030	2280	1707	-
99	547	350	-	510	480	2962	880	1086
101	971	380	1250	320	740	676	756	1034
102	414	2630	-	-	-	79	311	260
103	332	700	-	-	240	71	291	238
104	-	-	-	-	180	225	261	237
107	94	150	110	120	160	128	521	864
109	129	250	270	-	-	-	-	-
110	-	-	-	-	-	657	210	143
111	125	150	150	140	310	273	458	-
112	-	-	S	80	60	102	503	186
113	1711	200	310	170	500	-	-	-
114	655	570	1150	790	620	1277	1328	-
115	67	60	630	90	110	89	197	436
116	90	110	110	260	180	4068	1501	1912
119	300	130	430	160	3070	1677	1569	2692
120	-	-	-	170	80	120	103	148
121	145	80	130	120	120	127	1201	-
123	-	70	90	70	150	172	148	211
126	-	-	50	130	640	150	276	303
127	309	320	1340	1050	-	182	291	300
132	189	310	590	350	640	574	469	437
133	182	210	560	-	1250	233	484	723
134	85	110	S	230	610	237	-	-
135	2384	3520	3280	-	-	-	-	-
136	331	560	340	590	620	-	149	84
137	386	1820	760	540	520	1605	-	-
139	1170	840	-	190	300	136	143	154
141	-	-	60	62	140	64	81	70
142	409	60	70	99	300	135	354	493
143	72	200	70	610	480	827	-	-
145	-	110	70	50	80	263	145	3883
146	57	360	380	770	390	669	975	1603
147	-	-	740	110	500	718	259	374
148	727	1840	240	1300	3320	3054	804	1100
150	225	2570	0	80	120	140	207	275
151	42	70	80	120	150	123	459	-
152	1143	5920	150	-	-	-	-	-
155	752	280	350	410	590	473	-	-
157	-	-	110	-	50	67	59	172
158	-	-	-	-	160	563	457	394
159	209	120	100	120	290	265	209	177
160	-	-	70	150	1340	923	322	666
161	307	230	260	-	-	101	112	146
162	-	-	60	70	140	96	151	51
163	-	-	220	230	-	-	-	-
164	-	-	50	90	370	50	50	46
168	919	980	6670	-	-	67	189	359
173	-	-	-	280	248	226	216	273
177	-	-	550	-	-	-	-	-
180	636	610	3290	-	-	-	-	-
183	299	220	360	920	2300	1111	657	-
185	1371	930	930	460	930	-	521	337
187	115	-	-	-	-	-	-	-
999	449	507	494	519	612	760	661	523

Key S = Sour sample
- = No information available
999 = Herd average

Appendix XXI Farm 5 Visit 16.07.91

Results of Isolation Tests on Milk Samples and Skin Swabs

	Milk Samples						Skin Swabs				
Cow	Blood Agar Staph	Edwards Agar Strep	Todd Hewitt Broth	Inulin Broth	Inulin Serum Agar	Hippurate Broth	Edwards Agar Strep	Todd Hewitt Broth	Inulin Broth	Inulin Serum Agar	Hippurate Broth
2	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
4	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
5	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
6	+	+(+)	+	Y48	Y48	-	+(+)	+	-	Y48	-
7	+	-	ND	ND	ND	ND	+(+)	+	+(-)	C	ND
12	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
14	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
15	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
23	+	-	ND	ND	ND	ND	+(+)	+	+(-)	+(-)	ND
24	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
25A	+	+(h)	ND	ND	ND	ND	+(+)	+	+(-)	+(-)	ND
25B	+	+(+)	+	-	-	ND	-	ND	ND	ND	ND
27	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
29	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
30	+	+(+)	+	Y48	Y24	-	-	ND	ND	ND	ND
31	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
34	+	+(+)	+	-	-	ND	-	ND	ND	ND	ND
35	+	+(+)	+	-	-	ND	-	ND	ND	ND	ND
37	+	+(+)	+	-	Y24	-	+(+)	+	+(-)	Y24	-
41	+	+(+)	+	+(-)	-	ND	+(+)	+	+(-)	Y24	-
44	+	+(-)	ND	ND	ND	ND	+(-)	ND	ND	ND	ND
45	+	-	ND	ND	ND	ND	+(+)	+	+(-)	Y24	-
46	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
47	+	+(+)	+	+(-)	Y24	-	+(+)	+	-	Y48	-
48	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
50	+	+(+)	+	Y48	Y48	-	+(+)	+	Y24	Y24	-
51	+	+(-)	ND	ND	ND	ND	+(+)	+	Y24	Y24	-
52	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
53	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
54	+	-	ND	ND	ND	ND	+(+)	+	+(-)	Y48	-
56	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
60	+	-	ND	ND	ND	ND	+(+)	+	-	Y48	-
61	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
62	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
63	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
65	+	+(h)	ND	ND	ND	ND	+(+)	+	+(-)	Y48	-
66	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
67	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
69	+	+(h)	ND	ND	ND	ND	+(+)	+	-	Y48	-
74	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
77A	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
77B	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
78	+	-	ND	ND	ND	ND	+(-)	ND	ND	ND	ND
79	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
80	+	+(+)	+	Y24	Y24	-	+(+)	+	+(-)	+(-)	ND
81	+	+(+)	+	+(-)	+(-)	ND	+(-)	ND	ND	ND	ND
82	+	+(-)	ND	ND	ND	ND	+(+)	+	+(-)	Y48	-
84	+	+(+)	+	Y48	Y48	-	+(-)	ND	ND	ND	ND
85	+	-	ND	ND	ND	ND	+(+)	+	Y24	Y24	-
86	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
88	+	-	ND	ND	ND	ND	+(+)	+	+(-)	C	ND
89	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
90	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
91	+	+(+)	+	+(-)	+(-)	ND	+(+)	+	Y24	Y24	-

Results of Isolation Tests on Milk Samples and Skin Swabs

	Milk Samples						Skin Swabs				
Cow	Blood Agar Staph	Edwards Agar Strep	Todd Hewitt Broth	Inulin Broth	Inulin Serum Agar	Hippurate Broth	Edwards Agar Strep	Todd Hewitt Broth	Inulin Broth	Inulin Serum Agar	Hippurate Broth
94	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
96	+	-	ND	ND	ND	ND	+(+)	+	Y48	Y24	-
100	+	+(-)	ND	ND	ND	ND	+(-)	ND	ND	ND	ND
101	+	-	ND	ND	ND	ND	+(-)	ND	ND	ND	ND
102	+	+(+)	+	Y48	Y48	-	+(+)	+	Y24	Y24	-
104	+	+(+)	+	Y48	Y48	-	-	ND	ND	ND	ND
105	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
107	+	-	ND	ND	ND	ND	+(+)	+	Y24	Y24	-
110	+	+(+)	+	-	Y48	-	-	ND	ND	ND	ND
115	+	+(+)	+	+(-)	Y48	-	-	ND	ND	ND	ND
116	+	-	ND	ND	ND	ND	+(+)	+	-	+(-)	ND
119	+	+(+)	+	Y24	Y24	-	+(+)	+	Y24	Y24	-
120	+	+(-)	ND	ND	ND	ND	+(+)	+	Y24	Y24	-
121	+	+(+)	+	Y48	Y48	-	+(+)	+	+(-)	+(-)	ND
124	+	+(h)	ND	ND	ND	ND	+(+)	+	+(-)	C	ND
125	+	-	ND	ND	ND	ND	+(+)	+	Y48	Y48	-
126	+	+(+)	+	+(-)	Y48	-	+(+)	+	-	-	ND
127	+	-	ND	ND	ND	ND	+(+)	+	+(-)	+(-)	ND
132	+	+(h)	ND	ND	ND	ND	+(+)	+	+(-)	C	ND
133	+	+(h)	ND	ND	ND	ND	+(+)	+	Y48	Y48	-
136	+	-	ND	ND	ND	ND	+(+)	+	+(-)	C	ND
139	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
141	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
142	+	+(+)	+	Y48	Y48	-	-	ND	ND	ND	ND
145	+	+	+	Y48	Y48	-	-	ND	ND	ND	ND
146	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
147	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
148	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
150	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
151	+	+(-)	ND	ND	ND	ND	+(+)	+	+(-)	Y48	-
157A	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
157B	+	+(-)	ND	ND	ND	ND	-	ND	ND	ND	ND
158	+	+(+)	+	+(-)	Y48	-	-	ND	ND	ND	ND
160	+	+(+)	+	+(-)	+(-)	ND	-	ND	ND	ND	ND
161	+	+(-)	ND	ND	ND	ND	+(+)	+	+(-)	+(-)	ND
162	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
164	+	+(+)	+	Y48	Y48	-	-	ND	ND	ND	ND
168	+	+(+)	+	+(-)	+(-)	ND	+(+)	+	+(-)	Y48	-
170	+	-	ND	ND	ND	ND	+(+)	+	+(-)	Y24	-
173	+	-	ND	ND	ND	ND	+(+)	+	Y48	Y24	-
185	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
B2	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND
B3	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
B4	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
B5	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
X	+	-	ND	ND	ND	ND	-	ND	ND	ND	ND
Y	+	+(h)	ND	ND	ND	ND	-	ND	ND	ND	ND

Key

+ = Growth
- = No growth
(h) = Beta haemolysis
(+) = Aesculin hydrolysis
(-) = No aesculin hydrolysis (Edwards agar) or inulin fermentation (inulin broth/agar)
Y24/Y48 = Inulin fermentation within 24/48 hours
C = Contamination
ND = Not done

Appendix XXII Farm 5 Visit 16.07.91

Results of Antimicrobial Susceptibility Tests (Oxoid) on Representative Staphylococcal Colonies from Milk Samples

(Two or more colonies taken from some milk samples)

Cow	Ampicillin PN 10µg	Chloramphenicol C 10µg	Lincomycin MY 2µg	Oxytetracycline OT 30µg	Penicillin G G 10units	Streptomycin S 10µg	Cotrimoxazole SXT 25µg	Amoxycillin AML 25µg	Amoxycillin + Clavulanic Acid AMC 30µg	Cloxacillin OB 5µg	Cefuroxime CXM 5µg	Erythromycin E 10µg	Neomycin N 10µg	Novobiocin NV 5µg
14	SS	SS	R	SS	SS	S	R	SS	SS	S	S	SS	S	S
14	SS	SS	R	SS	SS	S	S	SS	SS	S	SS	SS	S	S
27	SS	S	S	S	SS	S	R	SS	S	S	S	S	S	R
27	SS	S	R	S	SS	S	R	SS	SS	S	R	S	S	S
67	SS	S	S	S	SS	S	S	SS	SS	SS	SS	S	S	S
67	SS	SS	S	S	SS	S	S	SS	SS	SS	S	S	S	R
88	SS	SS	SS	SS	SS	SS	R	SS	SS	SS	S	S	S	R
88	SS	SS	S	S	SS	S	R	SS	SS	SS	SS	SS	S	R
107	SS	SS	SS	SS	SS	S	R	SS	SS	SS	SS	SS	S	S
107	SS	SS	S	SS	SS	S	SS	SS	SS	SS	S	S	S	S
121	SS	SS	S	S	SS	S	S	SS	SS	S	S	SS	S	R
121	SS	S	S	S	SS	S	R	SS	SS	SS	SS	SS	S	S
121	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS
146	SS	SS	S	S	SS	SS	R	SS	SS	SS	SS	SS	S	R
146	SS	SS	S	S	SS	SS	R	SS	SS	S	S	S	S	R

Key SS = Area of susceptibility > 1.5cm diameter
S = Area of susceptibility < 1.5cm diameter
R = No susceptibility

Cotrimoxazole = Sulphamethazole + Trimethoprim

Appendix XXIII Farm 6 SMRA Milk Somatic Cell Counts (x 1,000 cells per ml)

Cow No.	Date of Calving	Lactation	04.07.90	23.09.90	14.10.90	09.12.90	07.01.91	05.02.91	05.03.91	06.04.91	29.05.91
1	13.12.90	8	319	-	-	-	53	95	1983	149	197
3	11.07 90	8	-	134	115	249	617	608	550	812	583
7	29.07 90	8	-	1128	2530	2360	5419	-	-	-	-
9	15.08.90	8	-	30	144	127	246	167	256	190	498
10	21.09 90	8	387	-	46	45	45	93	140	114	426
18	22.03 90	7	14	30	154	94	113	163	-	-	120
25	20.09.90	7	-	-	1011	145	65	91	424	132	221
31	16.10.90	7	-	-	-	433	682	2379	-	-	-
33	22.07.90	7	-	10	32	34	79	72	93	173	174
38	-	-	1099	1215	-	-	-	-	-	-	-
45	28.08.90	6	-	99	428	189	269	296	251	539	180
54	30.09.90	5	454	-	2300	195	445	508	474	-	-
56	19.07.90	5	-	101	237	498	78	463	332	259	460
59	02.09.90	5	-	13	34	44	74	124	138	138	177
60	15.09.90	1	2072	-	407	669	1282	942	978	810	584
61	14.07.90	4	-	137	174	48	81	97	202	-	-
63	05.09.90	4	-	254	129	107	192	165	248	357	167
64	17.07.90	4	-	13	30	428	497	223	650	-	-
65	30.07.90	4	-	20	36	37	101	95	135	127	180
66	03.08.90	4	-	405	266	83	156	179	396	-	-
67	24.07.90	4	-	311	468	262	794	641	553	637	415
68	08.07.90	4	-	33	42	186	1166	291	266	-	-
69	16.11.90	4	283	-	-	87	166	267	536	286	253
70	08.09.90	4	1080	102	130	49	94	71	133	103	212
71	19.09.90	4	617	-	326	70	60	41	53	60	-
72	25.08.90	4	-	59	156	76	119	80	105	225	-
74	12.07.90	4	-	361	324	358	833	234	259	-	-
75	25.07.90	4	-	16	45	37	78	43	80	76	86
78	17.07.90	4	-	369	252	176	268	566	296	255	-
79	22.08.90	4	255	111	119	227	744	201	264	270	219
81	15.07.90	4	-	377	321	342	390	350	388	584	-
83	08.08.90	4	-	447	428	1087	734	604	1196	761	915
85	01.08.90	4	-	273	62	76	401	144	1386	142	-
87	18.07.90	3	-	56	44	93	138	189	183	114	-
88	24.10.90	3	134	-	-	25	48	44	49	52	76
89	10.09.90	3	296	31	127	50	41	23	90	138	119
90	17.07.90	3	-	48	43	65	118	134	110	-	-
91	24.08.90	3	-	12	28	35	71	164	206	204	222
92	27.07.90	3	146	26	42	55	74	96	139	-	-
94	16.07.90	3	-	18	40	57	69	107	167	233	-
95	18.11.90	3	1279	-	-	41	46	41	486	-	-
96	11.09.90	3	129	36	359	49	73	141	251	381	-
98	26.07.90	3	-	296	238	423	850	246	374	366	283
101	14.07.90	2	-	70	110	74	71	130	137	118	-
102	25.09.90	3	156	-	44	46	51	41	69	121	136
103	21.07.90	2	-	115	143	922	S	465	98	-	-
106	28.07.90	2	-	34	67	129	411	471	774	-	-
108	-	-	-	103	101	-	-	-	-	-	-
110	12.08.90	2	-	51	126	47	94	106	95	90	-
111	07.07.90	2	-	139	723	178	248	341	261	501	-
112	10.10.90	2	94	-	-	101	425	146	162	191	145
114	19.07.90	2	-	26	43	60	79	77	127	478	-
115	11.07.90	2	-	3	32	36	S	42	51	47	59
116	26.09.90	2	92	-	42	34	69	48	58	81	99
117	23.07.90	2	-	465	369	740	404	563	418	-	-
118	29.07.90	2	-	22	47	48	119	103	402	-	-
119	22.08.90	2	-	36	52	33	35	46	58	58	-
120	07.07.90	1	-	711	236	106	S	138	163	149	-
121	10.07.90	1	-	42	44	35	45	52	46	49	-
122	02.08.90	1	-	26	61	63	48	46	48	42	-
123	27.07.90	1	-	21	32	55	53	43	66	408	250
124	28.07.90	1	-	20	45	37	44	110	108	79	53
125	28.07.90	1	-	230	354	171	429	307	404	263	-
126	13.07.90	1	-	39	55	164	217	117	285	247	-
127	26.07.90	1	-	17	89	32	65	39	53	52	-
128	25.07.90	1	-	64	46	56	109	71	73	70	-
129	22.09.90	1	-	-	29	46	53	39	30	38	323
130	16.07.90	1	-	35	51	37	47	41	42	39	-
136	17.07.90	2	-	25	48	40	226	75	93	63	-
999			554	165	236	209	217	173	199	223	238

Key

S = Sour sample
- = No information available
999 = Herd average

Appendix XXIV Farm 6 Visit 30.07.91

Results of Isolation Tests on Milk Samples

Cow	Date of Calving	Lactation	Blood Agar Staph	Blood Agar Strep	Inulin Agar 1 (60 h)	API Test	Inulin Agar (24 h)	API Test
1	13.12.90	8	+	+(h)	1	ND	3	ND
3	11.07 90	8	+	-	1	ND	4	ND
10	21.09 90	8	+	-	1	ND	4	ND
18	22.03 90	7	-	+(h)	1	ND	3	ND
59	02.09.90	5	-	+(h)	1Y	ND	4	ND
60	15.09.90	1	+	-	1	ND	0	ND
64	17.07.90	4	+	+	1	ND	4	ND
66	03.08.90	4	+	+	1	ND	4Y	NI
69	16.11.90	4	+	+(h)	2	ND	4	ND
70	08.09.90	4	-	-	1	ND	3Y	*S. faecalis*
74	12.07.90	4	+	-	1Y	ND	4	ND
78	17.07.90	4	+	-	1	ND	4	ND
81	15.07.90	4	+	-	0	ND	4	ND
89	10.09.90	3	-	+(h)	2	ND	4	ND
90	17.07.90	3	+	-	2	ND	4	ND
101	14.07.90	2	+	+	1	ND	4	ND
102	25.09.90	3	+	-	1	ND	4Y*	*E. avium*
103	21.07.90	2	+	-	1	ND	4	ND
110	12.08.90	2	-	-	0	ND	4Y	*S. faecalis*
112	10.10.90	2	+	-	2	ND	4	ND
114	19.07.90	2	-	+(h)	3	ND	4	ND
117	23.07.90	2	+	-	2	ND	4(1Y)	NI
119	22.08.90	2	+	-	1	ND	3	ND
120	07.07.90	1	+	-	3	ND	4	ND
121	10.07.90	1	+	-	1	ND	4	ND
122	02.08.90	1	+	-	2	ND	4	ND
124	28.07.90	1	+	-	1	ND	4	ND
125	28.07.90	1	-	+(h)	3	ND	4	ND
127	26.07.90	1	+	-	1	ND	4	ND
128	25.07.90	1	+	-	3	ND	4	ND
130	16.07.90	1	-	-	0	ND	1	ND
134	?	1	+	-	2	ND	4Y	*S. faecalis*
136	17.07.90	2	+	-	1Y	ND	4	ND
137	?	1	+	-	3Y	NI	4	ND
139	?	1	+	-	2	ND	4	ND
142	?	1	+	-	1	ND	4	ND
143	?	1	+	-	0	ND	4	ND
147	?	1	+	-	1	ND	4	ND
149	?	1	+	-	1	ND	4	ND

Key

Inulin 1 = Inulin serum agar (Bramley, King and Higgs, 1979)
Inulin 2 = Inulin agar (Bramley, 1982)
60h/24h = Maximum incubation time (hours)
1/2/3/4 = Degree of colony growth (1 = first streaking, 4 = all four streakings)
Y = Yellowing of agar ie inulin fermentation
* = Exception - 48 hours incubation
+ = Colony growth
- = No colony growth
(h) = Beta haemolysis
ND = Not done
NI = No identification
? = No information available

Appendix XXV Farm 7 Visit 29.10.91

SMRA Milk Somatic Cell Counts (x 1,000 cells per ml)

Cow No.	26.09.91	Cow No.	26.09.91
1	59	121	198
2	99	122	74
3	91	123	106
4	317	125	80
9	62	126	123
12	565	127	507
13	2077	129	268
14	3609	130	73
16	118	131	125
17	59	133	271
19	669	134	361
22	47	135	109
23	1454	138	268
26	920	139	115
30	228	142	739
32	56	143	876
33	193	144	127
34	606	145	88
35	42	146	100
38	253	148	136
39	183	149	315
41	188	150	100
42	603	156	499
43	1218	159	111
46	542	164	254
48	439	166	244
49	168	172	379
51	460	174	396
54	50	179	65
63	690	180	123
64	107	188	490
65	303	190	606
71	128	195	174
73	222	196	57
75	755	197	314
77	111	202	121
78	453	203	82
80	216	204	68
89	29	205	610
90	1015	207	84
92	716	208	240
94	67	210	130
96	323	214	39
98	129	215	114
99	1484	216	109
101	272	219	39
102	396	222	268
103	115	224	258
105	44	226	452
106	80	228	75
107	525	229	110
108	115	233	165
112	72	246	481
113	117	248	89
115	33	252	294
116	275	255	744
117	255		
120	93	999	289

Key 999 = Herd average

Appendix XXVI Farm 7 Visit 29.10.91

Results of Isolation Tests on Milk Samples

Cow	Inulin Agar	Blood Agar	Edwards Agar	Cow	Inulin Agar	Blood Agar	Edwards Agar
1	-	ND	ND	117	++	c	ND
2	-	ND	ND	120	-	ND	ND
3	++	c	ND	121	-	ND	ND
4	-	ND	ND	122	-	ND	ND
9	-	ND	ND	123	++	+	+(-)
12	-	ND	ND	125	++	lmu	ND
13	-	ND	ND	126	-	ND	ND
16	++	lmu	ND	129	-	ND	ND
17	-	ND	ND	130	-	ND	ND
22	-	ND	ND	131	-	ND	ND
26	-	ND	ND	132	-	ND	ND
31	-	ND	ND	133	-	ND	ND
32	-	ND	ND	134	-	ND	ND
33	++	lmu	ND	135	++	+	-
35	-	ND	ND	138	++	c	ND
38	-	ND	ND	139	-	ND	ND
39	++	c	ND	142	-	ND	ND
41	-	ND	ND	144	-	ND	ND
42	-	ND	ND	145	-	ND	ND
43	-	ND	ND	146	-	ND	ND
46	-	ND	ND	148	-	ND	ND
48	++	lmu	ND	150	++	lmu	ND
51	++	lmu	ND	155	-	ND	ND
54	++	+	-	156	-	ND	ND
58	-	ND	ND	159	-	ND	ND
63	-	ND	ND	160	-	ND	ND
64	++	-	ND	164	-	ND	ND
65	-	ND	ND	166	++	+	-
67	++	-	ND	169	++	+	+(-)
71	-	ND	ND	172	++	lmu	ND
73	-	ND	ND	174	-	ND	ND
74	-	ND	ND	179	-	ND	ND
75	-	ND	ND	190	-	ND	ND
77	-	ND	ND	195	++	+(h)	ND
78	++	c	ND	196	++	c	ND
80	-	ND	ND	202	++	c	ND
89	++	lmu	ND	203	-	ND	ND
90	-	ND	ND	204	++	lmu	ND
91	-	ND	ND	205	++	c	ND
92	-	ND	ND	207	-	ND	ND
94	-	ND	ND	208	-	ND	ND
95	-	ND	ND	210	-	ND	ND
96	-	ND	ND	211	-	ND	ND
98	-	ND	ND	214	++	c	ND
99	-	ND	ND	215	-	ND	ND
101	-	ND	ND	216	++	lmu	ND
102	-	ND	ND	219	++	c	ND
103	-	ND	ND	222	-	ND	ND
105	-	ND	ND	224	-	ND	ND
106	-	ND	ND	228	++	c	ND
107	-	ND	ND	229	-	ND	ND
108	-	ND	ND	233	++	lmu	ND
111	++	lmu	ND	246	-	ND	ND
112	-	ND	ND	248	-	ND	ND
113	++	c	ND	250	++	c	ND
115	-	ND	ND	252	++	+(h)	ND
116	-	ND	ND	255	-	ND	ND

Key
++ = Colony growth with inulin fermentation
+ = Colony growth (blood agar)
- = No colony growth
+(-) = Colony growth with no aesculin fermentation
lmu = Large mucoid colonies
(h) = Haemolysis
c = Contamination
ND = Not done

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