Studies of the infections in bovine teat canals, their epidemiology and role in the pathogenesis of mastitis

by

Derek Forbes
B.V.M.S., M.R.C.V.S.

Presented to the University of Reading for examination for the degree of Doctor of Philosophy.

October 1968

Acknowledgements

I wish to thank all those who have assisted in the work and preparation of the thesis. During the course of the work I have enjoyed the technical assistance of Mrs. J.E. Putley, Mrs. S. Lukey, Miss J. Birkett and Mr. H.E. Elsom, all of whose help I greatly appreciated. I am particularly indebted to Mrs. Lukey who prepared most of the figures in the thesis. Mrs. J. Scott prepared figures 1 and 2 and Mr. T. Sayer took and prepared the photographs of all figures. Mr. G. Coles and his staff are to be commended for their attentive management of the cows. I also wish to thank Dr. A.C. Baird-Parker who examined some bacterial cultures for me and Mrs. J. Lawrence who typed the manuscript.

Dr. G. Slavin supervised the work and I am grateful for his helpful and constructive criticism throughout. Finally, I wish to thank Staff Training Branch, M.A.F.F., for financial assistance and Mr. H.I. Field, Director of the Central Veterinary Laboratory, Weybridge for allowing this work to be done at that laboratory.

Contents

Tables

Tables

Figures

Figures (cont'd)

Figures (cont'd)

Introduction

Mastitis, an inflammatory disease of the mammary gland is of major importance in cattle in all milk producing countries. The development of mastitis is a dynamic process involving the external environment and management of the animals, their susceptibility to the disease and the efficiency of their defence mechanisms once the disease has established. There are numerous factors associated with the disease which have been incriminated as predisposing factors. Many of these are really just opinions, and consequently difficult to investigate experimentally. Even if this were possible, to investigate one in isolation from all the rest would be unlikely to provide an unequivocal result. From the view of satisfactory statistical analysis it is impossible, without very great resources, to investigate management, hereditary and all other factors as a whole. However, in the opinion of most active workers in the disease, and with a considerable volume of evidence to support them, there is one factor common to nearly all occurrences of the condition, that is the presence of an infective agent within the milk secreting tissue of the mammary gland. It has been proved experimentally that bacteria when introduced into the mammary gland can induce mastitis in susceptible animals. On the other hand it has not been proved that obvious malpractice, poor management and experimental stress can induce persistent mastitis without the presence of bacteria in the gland. The evidence is therefore that bacteria are essential to mastitis, and only when present may predisposing factors alter the severity of the disease.

Considerable evidence has been amassed showing that when bacteria enter through the teat canal into the mammary gland, particularly those pathogenic bacteria commonly present as intrammary infections, mastitis results. It is not however known how bacteria naturally pass through the teat canal. Adams and Rickeard (1963) summed up the position when they said:-

"The mechanism by which the bovine udder becomes infected has been ignored. Mastitis research has been largely directed toward control and prevention in which antibiotics, improved sanitation, correction of certain faults in milking techniques, and the use of the milking machine have been thoroughly investigated. This research has contributed little to the basic understanding of the manner in which micro-organisms invade the udder".

Mastitis of bacterial origin is very common and four principle pathogens cause the disease in countries where reasonable dairy hygiene is practised. They are Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis. Other bacteria frequently colonise the mammary gland but are seldom sufficiently pathogenic to the host to cause mastitis. Nevertheless their facility for invading the mammary gland should make their study rewarding in investigations concerning the method by which intramammary infections develop. Such bacteria are Corynebacterium bovis, Staphylococcus epidermidis and Micrococcus species. In addition to these bacteria, which commonly inhabit the mammary gland, numerous other bacteria if introduced into the gland can cause mastitis. In fact the evidence

available suggests that most bacteria, if introduced into the gland can cause mastitis, generally of a severe nature. The common mastitis pathogens are less virulent in that quarters with clinical mastitis, while of great importance probably represent a small proportion of the total numbers of glands affected. Subclinical mastitis, as detected by methods based on an elevated cell content of milk indicative of an inflammatory response to bacteria, has a much higher incidence. Many quarters which become infected with these predominant bacterial types may not develop clinical symptoms, or if they do the gland may have been infected for some time without any clinical signs. The animals response to intra-mammary infections with the common bacteria is in general less severe than it is to persistent infections with other bacteria

The question can then be asked "What have these bacteria in common and what is their common relationship with the mammary gland." Firstly, the bacteria are all non-motile (a factor which is significant when one considers possible modes of invasion through the teat canal), and secondly the bacteria are, with the possible exception of Streptococcus agalactiae all commensal organisms of the skin or capable of surviving on skin. Their relationship with the mammary gland may therefore be attributable to the embryonic development of the gland which is a specialised structure developed from the same embryonic tissue as the skin, the ectoderm. Considering the position of the bovine udder and the trauma and exposure to faecal and other contaminants, the fact that coliform and other organisms do not cause a higher incidence of mastitis is remarkable.

This adds weight to the observation that skin bacteria are most able to colonise the mammary gland.

In some preliminary work to that reported in the thesis Forbes and Hebert (1968) showed that in one dairy herd the majority of new intramammary infections of Micrococcaceae were preceded by a period when the bacteria were localised in the teat canals. Often the period was of some weeks duration, while in other teat canals, infections, after persisting for some time never penetrated into the mammary glands. Structurally teat canals are invaginations of skin and bacteria resident in them are skin infections.

The very close proximity of infections in teat canals to the interior of the mammary glands makes the study of these infections of interest in investigating the pathogenesis of mastitis. This thesis is an attempt to do this and the review of the literature which follows is primarily concerned with this.

The bacteriology and epidemiology of the common intramammary infections, and the structure and function of the teat canal will be discussed. Other factors which may participate in the pathogenesis of the disease are only discussed where they are directly concerned with bacterial penetration into the gland proper and the dynamics of the immediate result of the invasion.

1. The Anatomy, Histology and Histochemistry of the bovine teat and teat canal

The mammary glands develop from embryonic ectoderm. First a mammary bud grows which then invaginates. In the bovine foetus one

mammary bud is present for each one of the four mammary glands. The invagination of the bud forms a structure called the primary streak, in which fissures develop first at the proximal end of the streak. The fissure grows from the proximal end and extends distally to form the teat sinus. The embryonic teat canal is the most distal structure and a cornified layer of cells persist at the apex of the teat bud throughout foetal life. From the proximal end of the primary streak, secondary and tertiary outgrowths develop which will form the alveoli, the milk secreting tissue of the functioning udder. (Turner 1930 and 1939, Foust 1941).

The mature teat is covered by stratified squamous epithelium in which six layers were recognised by Mankowski (1903) and are described below. The stratum germinitivum is a single layer of cells which multiply rapidly and from which the other layers develop. Resting on this layer is the stratum spinosum which is a many celled layer. The surface of this layer has undulations (the rete pegs) corresponding to the ridges projecting into the epithelium from the dermis below. The stratum granulosum is composed of flattened cells which still retain their nucleus whereas the next layer, the stratum lucidum has flattened cells devoid of nuclei. The stratum corneum consists of very thin epithelial cells and these slough off the skin by abrasion. These layers consitute the epidermis which overlies the dermis. The latter may be subdivided into two layers, a deep reticular layer which is interlaced by coarse fibres forming a loose connective tissue, and a superficial papillary layer in which finer bundles of connective tissue form a loose network. The papillary

surface presents numerous conical elevations, the papillae of the dermis (rete pegs) which correspond to depressions in the inner layers of the epidermis (Turner 1933).

The skin of the bovine teat differs from the skin of other parts of the bovine body in that it does not have any sebaceous or sudoriferous glands, neither are there any hair follicles present, (Kitt 1882, Venzke 1940, Adams, Rickard and Murphy 1961). The teat skin invaginates at the teat orifice and lines the teat canal dorsally to the junction of the teat canal and teat cistern, where the epithelium changes to a double layer of columnar cells. At the junction the epithelium forms a series of folds which together form a structure called Furstenburg's rosette. The stratified squamous epithelium in the teat canal presents numerous longitudinal folds (Venzke 1940) so that only a star shaped lumen is seen in a cross section of the closed canal (Turner 1930). Murphy and Stuart (1955) described these folds in the canal and also showed that a wax like material completely filled the lumen when the canal is closed. Adams Rickard and Murphy et al (1961) studied the epithelium of the teat and teat canal and found that there were essential differences between the two. In the teat canal the stratum granulosum was markedly widened, tonofibrils in this layer were more numerous as were the numbers of keratohyalin granules, the rete pegs were wider and longer and the stratum corneum was much thicker.

Although the teat skin and teat canal are devoid of glands which are typical of other areas of skin, Weber, Wyand and Phillips (1957) found accessory glandular tissue in two teat canals of the 44 they

examined. The glands lay lateral to the canal and had ducts leading into the canal, which were lined by stratified squamous epithelium. Histological examination of the glands suggested that the glands were rudimentary alveoli which appeared to have some secretory activity. Johnston (1938) reported a similar structure in one of the 324 teats he examined. Rickard and Murphy. Helmboldt, Jungher and Plastridge (1953) and Adams ~~et al~~ (1961) did however find evidence of secretory cells in the teat canal which they thought to be sebaceous cells. Using histochemical techniques Adams showed groups of cells in the stratum granulosum which had an affinity for Sudan Black stain in their cytoplasm. These cells extended into the keratinised layer (the stratum corneum) in a narrow zone two to three cells wide and large lipid droplets were present at the junction with this layer. The authors interpreted these findings as evidence of lipid production in the stratum granulosum of the teat canal. They postulated that lipid migrated to the superficial epidermis in between epidermal cells and not in a specialised duct. Lipid production was most evident in or around the teat orifice and at the squamocolumnar junction. More specific histochemical tests aided by acetone extraction showed the lipid material consisted of phospho-lipids, triglycerides and free fatty acids. The material was thought to be comparable to sebum produced by sebaceous glands in other skin areas. Cytochemical techniques to study the metabolic activity of structures thought to be Golgi elements or mitochondria showed them to be concerned in the production of fatty substances in the lipid producing cells, protein in keranitising cells and both lipid and protein in cells they referred to as pseudosebaceous cells. Glycogen was also found in

the teat canal epithelium where it was localised in the stratum spinosum and basal layers of the stratum granulosum.

Hubben, Morse and Medley (1966) investigated the histochemistry of teats whose susceptibility to challenge with Streptococcus agalactiae was known. Differences were noted between susceptible and resistant teats but the methods did not allow their significance to be determined. They did however demonstrate glycogen and dehydrogenase activity in the stratified squamous epithelium lining the streak canal which they thought indicative of lipid synthesis.

The teat canal functions as the milk outlet and acts as a sphincter by involuntary muscle and elastic tissue which surround the canal. Christ (1905) and Kamm (1925) described a longitudinal muscle layer under the epithelium of the papillary eminences in the region of the teat canal, outside of which there is a circular layer which forms the muscular sphincter papillae. The muscles of the teat canal sphincter are controlled by the sympathetic nervous system. The elasticity of the teat canal may be supported by small columns of epithelial cells which extend from the papillae into the stratum spinosum. These structures were described by Mankowski (1903) as "Marksaulchen" and he suggested that they and the papillae give greater elasticity and firmness to the epithelium of the teat canal.

Part II

The penetration of bacteria through the teat canal

A. Anatomical factors.

i) Patency of teat canal

ii) Shape and size of the teat and teat canal

B. Suction during milking

C. Growth through the teat canal.

D. Capillary Action.

Part II

The penetration of bacteria through the teat canal

Mastitis is usually associated with the growth of bacteria in the milk secreting tissues of the udder. Numerous experiments have shown that bacteria, when inoculated through the teat canal into the teat sinus may become established and possibly cause mastitis. It is possible that invasion of the udder *via* the blood stream occurs, indeed one worker demonstrated this and it is likely that this is the mechanism by which *Brucella abortus* establishes infection in the gland. Nevertheless the main portal by which bacteria gain access to the mammary gland is by passage through the teat canal. On reaching the milk and milk secreting tissues the host defence mechanisms act to combat the bacterium which results either in the elimination of the infection, or if they fail to its persistence. In the latter case the virulence of the organism and the susceptibility of the host determines the severity of mastitis induced. Intramammary factors therefore can only act once invasion of the gland has occurred. The mechanism by which bacteria cross the teat canal is not understood but according to Plastridge (1958) "there is strong evidence showing that invasion of the gland is controlled largely by the characters of the teat canal".

There is great difficulty in determining the time of bacterial penetration into the gland. Early workers relied on the development of clinical mastitis as an indication of bacterial invasion. How

More accurate recognition of mastitis has shown that sub-clinical infections are very common and mammary glands that may be thought to be normal and uninfected are not in fact so. The interval between bacterial invasion and evidence of clinical inflammatory response may be of considerable duration or not occur at all, even when caused by recognised pathogens (Forbes and Hebert 1968).

As most intramammary infections are of non-motile bacteria there must be either a physical basis by which they are carried across the teat canal or they must grow through the teat canal. The review of the literature in this section is solely concerned with an examination of that work which relates to the teat canal and possible mechanisms by which bacteria pass through it.

Part II (2)

A. (i) Patency of the Teat Canal

Patent teat sphincters have been suspected by many workers to allow easy bacterial penetration into the udder. However, the term patency of the teat canal should be defined. Some teat sphincters are frankly incompetent and allow milk to trickle from the teat when the gland is full of milk. Davis (1935) noted this and suspected that such an occurrence, by creating a direct route from the udder to the exterior might allow bacteria to enter the gland. However, most teats, which by the ease with which they may be milked are termed patent, do not allow escape of milk unless the gland is ready to be milked, "let down" having been stimulated. It is obvious therefore that there are degrees of patency and that the canal under normal physiological control, prevents the escape of milk.

The patency of the teat canal is usually estimated by the ease with which milk can be drawn from the teat or by the rate of milk flow when machine milked. The teat orifice is controlled by the teat sphincter muscle and therefore teat patency depends on the size of the orifice and the extent to which it is controlled by the sphincter.

Meyer-Golling (1960) compared the histology of the teat canals from easy and hard milking cows, and found that the structure of the teats could be related to ease of milking. Regarding patency he showed there was a relationship between the number of epithelial folds in the teat canal and ease of milking. Easily milked cows in general had double the

number of folds to those in hard milking cows. There was no relation in the size of the sphincter muscle with ease of milking, in fact difficult milkers had less circular muscle in the sphincter than had normal cows. These two observations make it evident that teat canals which allow rapid milk flow are easily dilatable and hence must have adequate powers of contraction between milkings.

Espe and Cannon (1942) attempted to evaluate the difference in tone of the teat sphincters of easy and of hard milking cows. They used a sphygmomanometer applied to the teat proximal to a clamp at the base. Readings were made when pressure in the bladder first caused milk to flow from the teat. The method was restricted because of the impossibility of obtaining standard physiological conditions. Application of the clamp sometimes caused leakage of milk, even in hard milkers. The results could not be duplicated and the authors could not satisfactorily explain the inconsistencies. They subsequently stated that 100 mm Hg pressure was sometimes necessary to start milk flow but that the same rate of flow could be maintained by 50 mm Hg. pressure. They thought that the sphygmonanometer may cause the folds of Fustenburg's rosette to occlude the aperture, although it is extremely possible that the difference was due to a relaxation of the teat sphincter associated with a "let down" stimulus. Their work showed no definite trend between patency of the teat canal and susceptibility to mastitis.

Gorodetskaya (1967) investigated the physiological control of the teat sphincter and found that sphincter tonicity and intramammary pressure

are closely related under normal milking conditions. During milking in full udders the sphincter is in a slack state whilst, between milkings it is contracted. She also found that the cavity of the mammary cistern functions as the receptor zone for the regulation of sphincter tonicity, and together these control the intensity and character of milk extraction. The rate of milking was related to the sphincter tonus during milking, which was 40-80 mm Hg for milking times of 4-5 minutes, and 40-360 mm Hg for milking times greater than 15 minutes.

Pounden & Grossman (1950) found that ante and post mortem teat pressures sufficient to prevent leakage of milk were very similar. This suggested that nerve control of the muscle was unlikely to be the only mechanism in the closing of the spincter. Thiel, ~~et al~~ Clough, Westgarth and Akam, (1966) showed that at pulsation rates above 50 cycles per ~~sec.~~ min the teat sphincter was almost totally passive because of its inability to contract with a speed comparable to the vacuum pulsations. At slower pulsation rates the milk flow was the same at the end of 1.5 sec. milk flow as at 0.5 min. at zero pulsations. This would suggest that the closing mechanism of the teat sphincter cannot close the orifice during milking and closure is dependent on the liner wall movement giving lateral pressure at the teat apex.

Pier, Schalm and Hage (1956) showed by radiography that the rubber liner collapsed below the end of the teat squeezing the tip. Ardran, Kemp, Clough and Dodd (1958) showed by cineradiographs that milk flow stopped during a pulsation cycle when the collapsing liner was about one half the normal width below the teat and restarted as soon as the liner began

to open. As a negative pressure is always present at the teat end, these observations must show that the teat orifice is closed during the rest phase of milking, by lateral pressure sufficient to cause cessation of milk flow before complete collapse of the liner. As soon as this pressure is released as the liner begins to reopen, milk flow restarts.

Gorodetskaya (1967) states that the teat sphincter is in a slack state during milking although as she did not report her methods of determining this, it is not known if any pulsation of the sphincter was observed. Using a milk flow recorder Phillips (1965a) showed that the characteristics of the teat orifice are markedly different at the commencement of milking to those once the flow has been established. He likened the teat sphincter to a heavily damped spring which gains in diameter extremely slowly during the opening process resulting in a gradual increase in milking rate for a period at the commencement of milking. Thiel et al.(1966) thought that pulsation did occur during milking at pulsation rates lower than 50 cycles per ~~sec~~ min. They thought the decrease in milk flow from the initial high rate during a pulsation cycle was probably due to contraction of the sphincter muscles after being stretched open by the pressure difference across the teat canal. They also thought at shorter cycles the time available for contraction of the sphincter was also shorter, and consequently a lower residual force was exerted by the muscle when next expanded. This, they used to explain progressively higher milk flow rates at increasing pulsation rates. However, it is a character of unstriated muscle that it is sluggish in action, and muscles tend to show slow spontaneous rhythmic contractions

(Winton and Bayliss 1955). It is more probable that the changes in flow described by Thiel et al.were influenced by elastic fibres in the sphincter. Whittlestone and Olney (1962) concluded from their experiments investigating milking rates at different periods of one milking that the teat spincter becomes slightly stretched throughout milking.

Witzel (1967) who measured contraction of the teat canal by insering minute balloons in the teat canal did record pulsations occurring at oestrus and anoestrus but the changes in muscle tone in the spincter were very slow. It is extremely unlikely that the tone of smooth muscle can change sufficiently rapidly to keep pace with machine milking at the pulsation rates normally used and the teat canal is probably passively relaxed during milking.

Clarke, Dodd and Foot,
Baxter, ~~et al~~ (1950) showed that the rate of milking is governed by level
the vacuum/and effective size of the teat orifice during milking. Machine milking rate was therefore an objective measurement of teat patency. They found that the maximum rate of milking by both teat cup and teat cannula increased with increasing level of vacuum. The rate of increase in the teat-cup milking was greater than in the cannula milking which suggested that the teat orifice was stretched open at high vacuum levels. Andreae (1958) devised an instrument to measure the extendibility of teat canals using a micrometer screw gauge to give constant pressure of distension. He found that the maximum rate of milk flow per minute increased with increasing extendibility of the teat canal while the duration of milk flow decreased. In other words teats with easily extendible teat canals had high flow rates and milked out quickly.

He did not examine these characteristics relative to the incidence of mastitis. Nevertheless he did establish that rate of milking depends on the patency of the teat canal during milking. Other workers have investigated the relation between ease of milking and mastitis and their evidence is somewhat conflicting.

Udall, Johnson and Ferguson (1938) considered that if milk flows very easily from a teat on slight handling then it is most probably infected for in such quarters it is impossible to avoid invasion. McEwan and Cooper (1947) found a higher incidence of mastitis in quarters classified as easy milkers than in those classified as hard milkers. On the other hand Hughes (1953) concluded that teat patency was not of primary importance in preventing the entry of Streptococcus agalactiae although he thought it may be with other mastitis pathogens. Stuart and Lancaster (1949) could not establish a definite correlation between teat patency and susceptibility to infection but Davidson, Slavin and Stuart (1954) recorded that the one animal who became infected with Streptococcus agalactiae in a group milked under a rigid hygiene programme had slack teat sphincters. Her twin, who also had slack sphincters was the most susceptible animal in the other group, where milking machines were transferred directly from one cow to the next. Dodd and Neave (1951) grouped 94 first lactation heifers into five groups based on peak milk flow per minute and showed a strong correlation between the infection rate and high milking rates. This was true both during lactation and in the first dry period. The commonest infection was a coagulase positive Staphylococcus. They thought the most probable

explanation of the results was that bacteria may more readily gain entrance to the udders through larger or slacker teat sphincters.

Murphy (1944) estimated teat patency by exerting sudden lateral pressure to the middle of the teat with the thumb and first finger. He obtained circumstantial evidence that a trend existed towards a greater incidence of infection in quarters, the teats of which he judged to be patent than in those judged to be non-patent. In a later paper he and a co-worker (Murphy and Stuart 1955) examined machine milking rate and the susceptibility to artificially induced Streptococcus agalactiae infection. In those quarters which became infected there was no general relationship to the maximum rate of milk flow, although the fastest milking group had a much higher rate of infectability than the other groups, but this was due almost entirely to the records of two cows within the group. The difference between their work and that of Dodd and Neave (1951) may lie in the different intensity of bacterial challenge. Certainly the latter's work indicates that patent teats have an increased susceptibility to staphylococcal infection.

More recently Boge (1965) and Krejakovic et al (1967) by investigating the effect of milking on the occurrence of mastitis established that easily milked cows are more rarely affected than others. This may of course be due to different degrees of stress during milking.

If patency does influence the incidence of mastitis, and the evidence for this is equivocal, it is probable that the structure and functioning of the teat canal itself is important rather than the size of the aperture through the canal.

Part II (2)

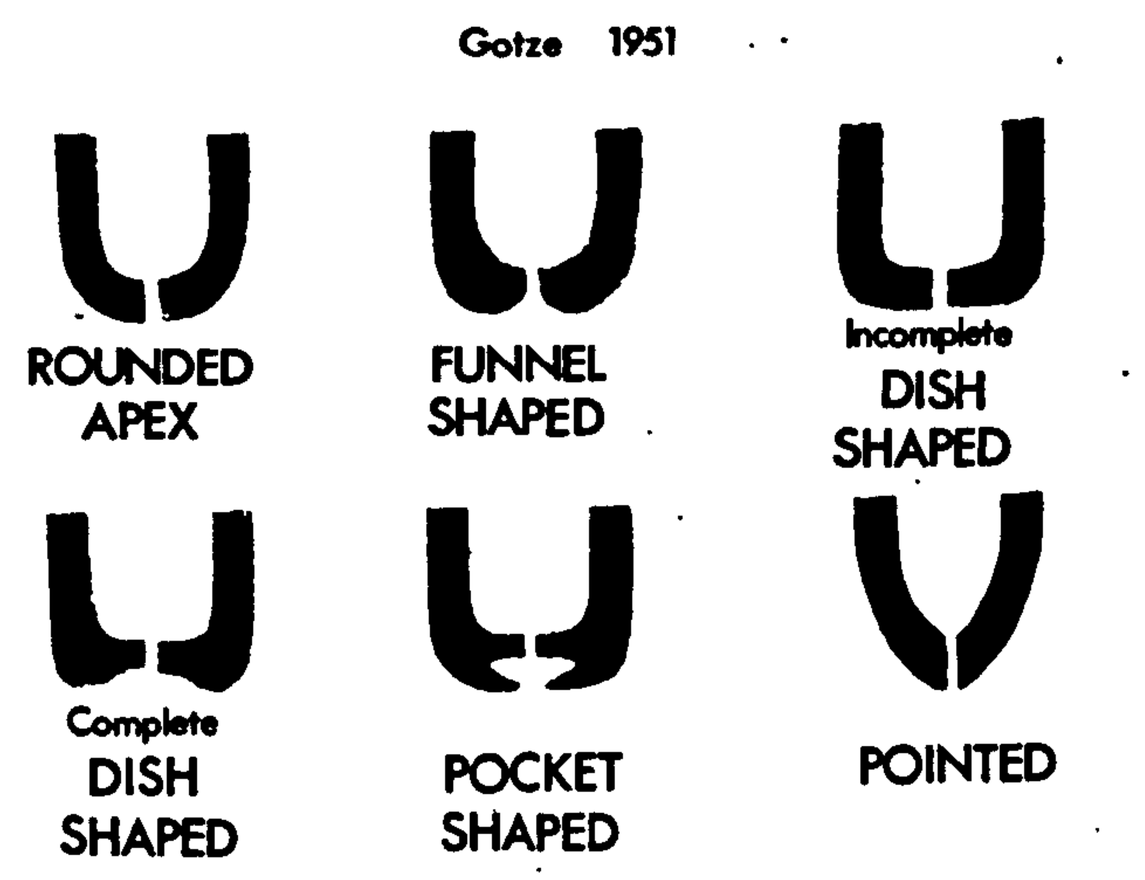
A. (ii) Shape and size of the teat and teat canal

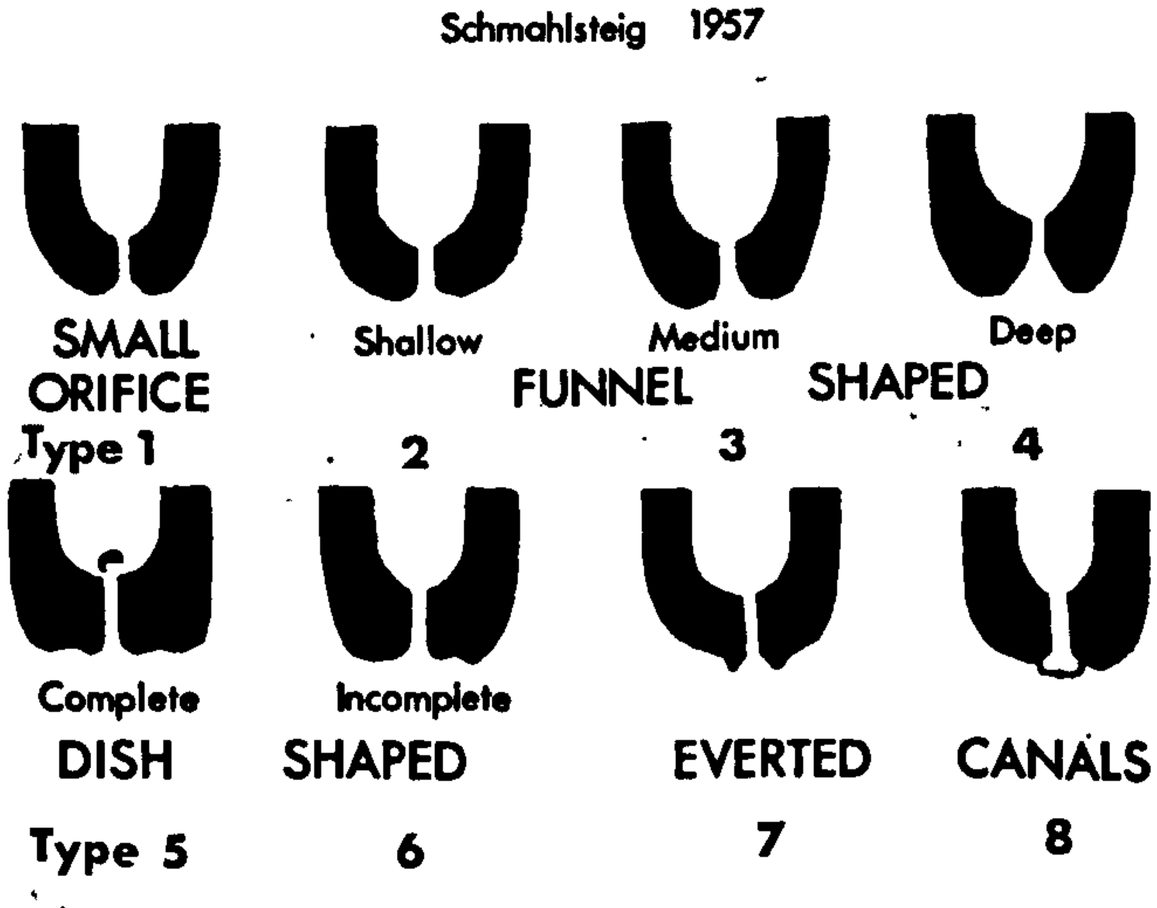
The passage of bacteria across the bovine teat canal into the teat sinus may be due to mechanical transfer and this may be facilitated by the size or shape of the teat and its canal. Several workers have investigated this and as in most other aspects of the susceptibility of the mammary gland to infection, the evidence is equivocal. The factors which have been investigated are the shape of the teat, particularly the end of the teat, and also the characters of the teat canal. Ilgmann (1933) examined the teats of 1,000 cows and related this to the incidence of mastitis. He found a marked difference in the incidence between cows with round or half round teat ends (approximately 8.5 per cent) when compared to dished, funnel or pocket shaped teat ends (approximately 20 per cent). McEwan and Cooper (1947) on the other hand found no evidence that the conformation of the free end of the teat or the presence or absence of eversion or erosion of the teat orifice exerted any influence on the susceptibility of the quarter to bacterial infection associated with mastitis. Later, Götze (1951) concluded that any teats with crater, plate or pocketed teat ends, and also pointed teats favoured the development of mastitis. He thought this was due to short teat canals and because the shape of the teat apex retained a drop of milk after milking providing a suitable medium for bacterial growth. He also stated that short, wide or slack teat sphincters are related to a high incidence of mastitis. Murphy and Stuart, (1955) however, found that in 6 cows infectability with Streptococcus agalactiae

was not related to the length of their teat canals. Kruger (1953) who asked if certain teat and udder shapes favoured the development of mastitis came to conclusion they did not. He could find no relationship between teat consistency, teat size, difficulty in milking or the shape and size of the teat end and mastitis. In contrast Jahnke (1954) recorded that teats with plate ends or inverted funnel shaped ends had a higher incidence of mastitis than other types. This was also reported by Boge (1965) who found the highest incidence of mastitis was in animals with plate shaped teat ends (42.6%) and those with funnel shaped teat canals (56.1%) compared to 30.2% incidence in normal round ended teats.

Schmahlsteig (1957) categorised the shapes of teat ends (See figure 1) into eight different types. The figure relates his nomenclature to that of other authors cited in this review. While his was a purely anatomical study Krejaković et al.(1967) related teats classified by his methods to their prevalence to mastitis. They found that teats of type one were most frequently infected with Streptococcus agalactiae and that there was a significantly higher incidence of mastitis in type 1 than in types 2 and 5. These three types were most common and comprised approximately 79 per cent of the teats in the survey.

It has been suggested therefore, by some but not all investigators that anatomical variations in the teat apex and the region of the teat canal orifice are associated with a high incidence of mastitis and these factors must therefore predispose the quarters to infection. Using a swab inoculation technique by which approximately 5×10^6 viable bacteria were placed in the external 3 mm of the teat canal, Murphy and Stuart





SHAPE OF THE TEAT APEX AND ORIFICE

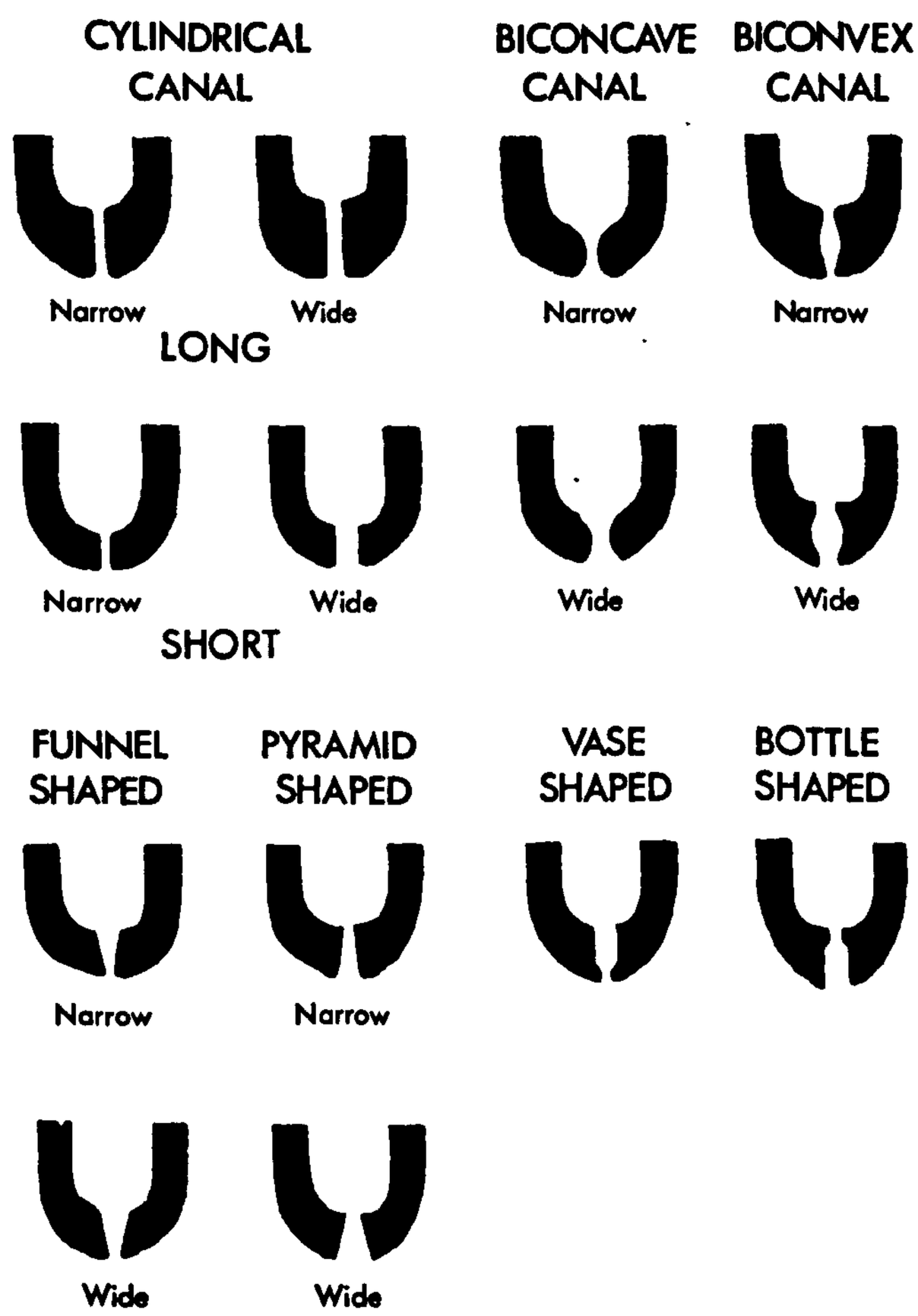
OF THE TEAT CANAL

Fig. 1

(1954a) noted a marked difference in susceptibility to infection in different cows. As the inoculation procedure was identical for each teat the authors concluded that it was largely a characteristic of the cow or quarter that determined whether infection resulted. In a later experiment (Murphy & Stuart 1954b) resistant cows were protected by parenterally administered penicillin during the period when the teat canals were inoculated, and were found still to be resistant. In contrast cows that had previously been completely susceptible, when protected by penicillin were more resistant to the infection than were the normally resistant animals. The inability of the bacterium to survive in the teat canals of susceptible cows longer than in resistant cows in these conditions, suggested to the authors that the teat canals of the susceptible cows were not so constituted physically as to be able to retain the organism (in a non growing state) for as long as the teat canals of the resistant cows.

Murphy (1959) reported the effect of mild stresses on the teat canal in relation to susceptibility to infection with Streptococcus agalactiae. The stresses imposed were, 1) swabbing the teat canal, 2) prolonged milking at high vacuum levels and 3) the removal of the soft keratin lining the teat canal. The first two did not cause any change in the resistance to infection or any histological changes whereas the third method caused a complete breakdown to resistance as intramammary infection always occurred when the teat canal was inoculated with the test organism. The resistance returned when sufficient time elapsed for the keratin to be renewed in the teat canals. These findings were interpreted to show that the removal

of the keratin resulted in a physical impairment of the teat canal closure but it was not thought that anything resembling the removal of keratin was responsible for naturally occurring infections. The physical constitution of teat canals has not been examined in the living animal but Johnstone (1938) and Schmahl[5]teig (195[7]) looked at the shape of teat canals (See Figure 2) at post mortem examinations. Both found that the circumference of the canal need not be uniform throughout its length. Arnold (1950) found 80.3% of the teats he examined had funnel shaped teat canals with the external orifice being narrower. Keratin in the teat canal, being derived from stratified squamous epithelium must be produced at a fairly constant rate and consequently must be removed at an approximately equal rate at milking. Dry cows often have a plug of the material in the teat canal which of course does serve to plug the canal effectively during the dry period. A personal observation of the teats of dry cows shows that the material often protrudes from the teat canal and this often breaks off and gradually reforms. This shows continuous production of keratin some of which may move upwards into the teat sinus. During machine milking the collapsing liner wall imposes lateral pressure on the teat canal, the degree of compression being progressively diminished from the orifice to the base of the teat (Pier ~~et al~~ 1956). Pounden ~~et al~~ (1950) Schalm and Hage, and Grossman and Walser (1966) showed that machine milking removes more keratin from the teat canal than hand milking or calf suckling yet McDonald and Witzel (1966) showed that calf suckling caused a markedly higher differential pressure across the teat canal than did machine milking. They described the active phase of calf suckling which 1) creates a vacuum at the end of



SHAPE OF THE TEAT CANAL

Schmahlsteig 1957

Fig. 2

the teat within the teat cavity and 2) produces pressure within the teat sinus by compression of the teat between the tongue and hard palate. They thought the base of the teat is occluded by the tip of the tongue, dental pad and hard palate causing a pressure increase within the teat sinus. This compression obviously cannot extend to the tip of the teat otherwise milk flow would be prevented by closure of the orifice. Thus the increased removal of keratin from the teat canal reported by Pounden et al. must be due to the difference in the mechanics of milk removal rather than the degree of suction. Lateral pressure on the walls of the teat canal may also cause keratin to be squeezed up the teat canal, particularly in funnel or hourglass shaped teat canals (see figure 2). The forces necessary for this were shown by Pier ~~et al.~~ Schalm and Hage (1956) who demonstrated that the liner collapsed below the end of the teat, exerting maximum pressure at its tip and which decreased progressively up the teat. Phillips (1965b) argued that thick walled liners being unable to fold round the end of the teat during the rest phase caused a pinching action at the tip. This action, apart from possibly forcing milk back up the canal may cause keratin to be moved upwards and if bacteria are present in the keratin this may be an important mechanism by which their transfer into the teat sinus is effected. Noorlander (1960) stated that much of the desquamated epithelial wax like mass coating the teat canal is probably removed by a malfunctioning milking machine. This was shown histologically in overmilked teats by Walser (1966). Johansson (1957) reported that pointed teats or teats with plate formed tips predispose to teat erosion which is a manifestation of damage to the keratinaceous lining of the

teat canal. He also reported that teat erosion was never found in teats with a funnel shaped entrance to the teat canals, a morphological character with Ilgmann(1933), Götze (1951), Jahnke (1954), Boge (1965) and Krejakovic-Miljkovic et al. (1967) all found associated with a high incidence of mastitis. In such teats it is possible that liner wall movement tends to cause inversion of keratin rather than eversion.

Noorlander and Schalm (1958) showed by a study of liner design that the magnitude of liner squeeze on the teat is greater with liners of large diameter. This may explain the findings of Dillon et al.(1967). They investigated the influence of wide and narrow milking machine inflations on udder health over a period of three years in 53 cows. The left half of each udder was milked with wide bore inflations while the right was milked with narrow bore type. Of the total number of quarters infected 43.2 per cent were milked with the narrow type while 56.8 per cent were milked with the wide bore type. While the incidence of infection in both groups is quite similar the higher level in the quarters milked by wide bore inflations may indicate an increased tendency to invasion of the mammary gland by bacteria in these quarters due to a higher lateral pressure on the teat canals.

Dodd, Oliver and Neave (1957) carried out an experiment to study the effect on udder health when cows were milked with moulded or extruded liner. The wide barrelled moulded liner was used at a relatively low tension whereas the narrower barrelled extruded liner was at high tension. The incidence of new infections were the same in each group although more

mastitis was found in the teats milked with moulded liners. There was no measurable effect on teat erosion. The degree of pinching at the tip of the teat must depend on the relationship between the diameters of the liner and the teat and the tension of the liner. Hickman (1964) found a striking trend of increased incidence of mastitis with increase in teat diameter. He also found that funnel shaped teats had a lower frequency of mastitis than cylindrical shaped teats. Thus it may be that mechanical stresses imposed by machine milking on teats with certain anatomical characteristics may cause movement of keratin within the teat canal, thereby impairing the efficiency of the canal and possibly introducing infection into the gland from the teat canal or external orifice of the teat.

Part II (2)

B. Suction during milking

It is suspected by many workers that bacteria are sucked into the udder during pressure changes which occur during milking. However, the evidence for this is somewhat equivocal. It was shown by immersing teats in carbol-fuchsin as the pressure on the teat was released after milking that the dye did not ascend more than half way up any teat canal, even in those teats which leaked (Davis 1935). This work was done on 13 cows which were slaughtered immediately after the experiment. A similar experiment by Espe and Cannon (1942) in which a suspension of barium was used instead of dye and where X-rays were used to detect the barium did not demonstrate any suction of the material into the teat canal. They did, however, show that barium paste was drawn into the lower teat canal after being applied to the teat orifice while the greatest possible/manual pressure was exerted without/actually allowing milk to escape.

These experiments provide evidence that suction into the teat canal may occur during milking. Little,(1937) was able to demonstrate that mastitis could be produced experimentally when teats under manual pressure were immersed in fluid cultures and the pressure released. He concluded that the bacteria had been sucked into the canal but later implied that the bacteria were sucked into the udder. His methods could not determine with accuracy the time at which bacteria reached the teat sinus. This ambiguity of terms regarding the teat canal and udder led Plastridge (1958) to suspect that "micro-organisms may pass through the teat canal during milking." The facts from the available

evidence are that micro-organisms may pass into the teat canal during milking but it is not proved that they pass into the udder.

McEwan and Samuel (1946) however did show that bacteria applied to teat ends during milking did contaminate the teat duct and reach the milk and the teat sinus. This was shown on post mortem examination immediately after the living animals had been milked by hand or machine milking. During hand milking the teats were immersed in culture and during machine milking cultures were sprayed onto the teat. This experiment has been criticised because a motile bacterium was used which may account for their penetration of the teat canal. Surprisingly a similar experiment has not been reported in which a non-motile mastitic pathogen was used. However, from published work it is not proven that non-motile bacteria pass through the teat canal during routine milking.

The physical conditions necessary for bacteria to be sucked through the teat canal have been demonstrated after machine milking by Petersen (1944) and Carulo and Marx (1962) in excised mammary glands. Petersen showed that at the end of milking when the orifice between the gland sinus and teat was closed the vacuum within the teat sinus was the same as that in the milk line. With each release of vacuum in the shell of the teat cups the vacuum disappeared within the teat which was attributed to the collapse of the rubber inflation compressing the teat. Carulo and Marx (1962) obtained similar results. They like Peterson, did not record a negative pressure within the gland sinus, but only in the teat cistern. More recently Witzel and McDonald (1964) conducted similar

experiments in vivo. They found that pressures within the gland and teat sinuses were similar when both contained milk. The pressure decreased during milking until nearing the end of milk flow a slight vacuum of 0 to 5 mm Hg developed within the udder. When milk flow ended, teat sinus vacuum rapidly increased to a maximum with each inflation dilation. A residual teat sinus vacuum of between 48 and 120 mm Hg persisted after inflation collapse. This extended into the gland sinus only to the extent of a vacuum between 8 and 35 mm Hg. The authors believed that the gland sinus does not exhibit marked pressure changes because of the closure of the annular ring at the end of milk flow. By ligating the teat canal after milking and then continuing milking they showed that no residual vacuum ensued. From this they concluded that vacuum changes within the teat sinus during milking result from two factors: "(1) dilation and collapse of the teat sinus and inflation concomitantly and (2) extension of the milk line vacuum through the teat canal into the teat sinus during inflation dilation". Thiel, Clough and Dodd (1965) queried their work regarding the existence of a residual vacuum within the teat sinus after milking. They argued that stress on the teat tissues and the weight of the milking machine cluster may cause an apparent residual vacuum particularly with the pneumatic recording devices which were used. They concluded "that the extension of the milking vacuum through the teat canal to the teat sinus is still doubtful". Nevertheless, they could not explain wholly satisfactorily the absence of a residual vacuum after ligation of the

teat canal when with a patent canal such a vacuum occurred. The results of Witzel's and McDonald's experiment make their suspicion valid, that the development of a residual vacuum after machine milking possibly contributes to the passage of bacteria through the canal. High residual vacuum has since been shown (Cowhig, Nyhan and O'Flaherty ~~and~~ Phillips, 1968) to be a measuring artefact due to the use of an open cannula in the teat sinus to record pressure changes. They also showed that the low residual vauum of 4-12 mm Hg disappeared as soon as the teat cups were removed and found this was probably due to the passage of small amounts of milk from the gland sinus. They also found that this high residual vacuum in the teat occurring when a measuring catheter was in the sinus, persisted indefinitely if the root of the teat was occluded manually. The aspiration of bacteria through the teat canal is therefore unlikely, (Cited by Thiel, 1968). Indeed, the possibility of a vacuum developing at all in a chamber with completely collapsible walls when surrounded by the atmosphere seems most improbable. It should be pointed out, however, that in these experiments a prerequisite for residual vacuum to develop was that milk flow must have stopped completely. Under normal milking conditions this constitutes extreme overmilking and as such is not recommended.

Nevertheless there are several reports of overmilking not influencing the severity or incidence of mastitis. Mochrié et al (1953, 1955) failed to demonstrate statistically significant differences in mastitis associated with varying vacuum levels or milking for double the normal milking times. Manunta, Minole and Maraugui (1966) machine

milked four cows for a total of 1,200 times and despite not using antiseptic precautions and milking for 10-15 minutes after cessation of milk flow found the only effect was a slight transient hyperaemia of the teat. Walser (1966) overmilked half of each udder of 16 cows over a period of seven weeks for 10 or 20 minutes. No clinically demonstrable changes in teats, udder or milk resulted although milk samples taken from the over milked quarters occasionally showed a rise in cell content.

Neave, Oliver and Dodd (1962) milked cows at 20 inches Hg negative pressure for 5 minutes longer than normal for 10 months. These procedures did not significantly increase the incidence of udder infection or the incidence or severity of mastitis, even though the teats were dipped in a broth culture of S. aureus before each milking. Bratlie (1966) on the other hand, showed that aftermilking lasting more than one minute is an important cause of mastitis. This examination was done on 1327 cows in 116 herds and was found to be significant at the 5 per cent level.

It is possible that certain physical conditions may be necessary for bacteria to be sucked into the gland. Wilson (1958) deduced that a fluctuating vacuum in machine milking may predispose to mastitis. A fluctuating vacuum is a consequence of an inadequate vacuum reserve and causes slow milking. (Stanley, Kesler and Bartree, 1962, Schmidt, Switzer, Guest and Guthrie, 1964). Nyhan and Cowhig (1967) who showed that a low vacuum reserve was associated with a high prevalence of mastitis, thought the fluctuating vacuum and consequent slowing of milking rate may provide the physical conditions necessary for bacteria

to gain entry to the teat cistern from the teat canal or exterior of the teat.

Nyhan (1968) discussed the possibility of this occurring during overmilking. He pointed out that the pressure pattern in the teat sinus during overmilking approximates that in the teat cup chamber. Consequently if the pressure in the teat cup chamber drops during the release phase the vacuum in the teat sinus drops accordingly. When the vacuum drops during the release phase the pressure at the outer end of the teat canal may be momentarily higher than in the teat sinus. He suggested that in such a dynamic situation small capillary movements may occur in the teat canal resulting in movement inwards of bacteria already in the canal. Thiel (1968) in discussing this phenomenon cites J.S. McDonald as having failed to demonstrate it. The forces necessary for suction of bacteria through the teat canal are therefore suspected but have not yet been demonstrated. Supporting evidence is however given by Wilson (1968). He showed that in two groups of cows, which were exposed to identical levels of Str. dysgalactiae, the group milked with a very unstable vacuum had a higher incidence of infection than those in the group milked with a stable vacuum.

The work of McDonald cited by Thiel (1968) has now been published. (McDonald and Witzel, 1968). They showed that after milk stopped flowing from all four teats the vacuum at the teat end was stable through each pulsation cycle. During milk flow the vacuum did fluctuate at the teat end. It is unlikely that the pressure changes described by Nyhan (1968) occur after milking.

Part II (2)

C. Growth of bacteria through the teat canal

Hopkirk (1934) was the first person to establish that bacteria grow within bovine teat canals of quarters whose glands may be uninfected. He argued that the infections may and no doubt frequently do extend into the teat sinuses, thereby causing mastitis. From his experiments he postulated "that cows having a duct flora of streptococci or staphylococci may eventually develop mastitis when the conditions lend themselves to further invasion of the sinuses and main gland". He also introduced organisms into normal teat canals in which five out of six became established. However an elevation of cell count occurred in the milk from these quarters, a feature which Hopkirk thought a consequence of a teat canal infection but which was almost certainly an intra-mammary infection in view of more recent work (Murphy and Stuart, 1954, Newbould and Neave, 1965[a] and Forbes and Hebert, 1968). Indeed he confirmed the presence of intramammary infections in two experimental cows although the numbers of bacteria were very low. Murphy and Stuart (1953[b]) introduced Streptococcus agalactiae into teat canals by the Hadley-Wisconsin swab technique. They demonstrated that in many quarters the bacterium died out without causing a gland infection and therefore considered that the teat canal had been positively shown to represent a barrier which can prevent the inception of mastitis. The bacterium was present in milk from these quarters for up to 6 milkings after

inoculation. In quarters which developed intramammary infection following the inoculations the bacterium was estimated to have been confined in the teat canal for up to 18 milkings following inoculation. From this they supposed that significant growth of the test organism occurred within the canal to the extent that the bacterium penetrated into the gland by growth through the canal, but thought more information was needed before such a conclusion could be reached.

Sharpe, Neave and Reiter (1962) recorded that eroded teat apices were particularly susceptible to colonisation by diphtheroids and coagulase negative staphylococci. They also reported that S. aureus may also occur at the site and persist for several months in eroded teat canals without necessarily causing intramammary infections. Beech and Forbes (1965) showed by comparing the bacteria isolated in normally drawn milk and milk taken directly from the teat sinus by a syringe, that all the common udder pathogens can colonise the teat canal. Staphylococcus aureus persisted for periods up to 18 weeks without penetrating into the gland. The teat canals in this study were normal and showed no evidence of erosion. There is published evidence therefore that bacteria commonly associated with mastitis can colonise and survive in the teat canal, often for quite considerable periods.

Murphy and Stuart (1953) however, who used Str. agalactiae found a marked difference in susceptibility between cows and individual quarters, a feature which they (Murphy and Stuart 1954) thought to be

due to a difference in the physical constitution of the teat canals. Newbould and Neave (1965c) who inoculated S. aureus into the outer 4mm of the teat canals also found a varying reaction between cows. A single inoculation resulted in either : no colonisation of the canal, temporary colonisation for up to 7 days, or in colonisation followed by intramammary infection. They argued that in those quarters from which the bacterium was not recovered at the first post inoculation milking, the staphylococci were killed by some bactericidal factor in the canal or died out for lack of some essential metabolite. This could not be applied to those instances where multiplication first took place and the bacterium subsequently died out, so it was thought that some factors which supported initial multiplication became depleted and the bacterium was then susceptible to bactericidal factors.

Murphy (1959), who showed that the efficacy of the teat canal barrier to infection was impaired by the removal of keratin from the canal, led Adams and Rickard (1963) to analyse keratin for evidence of antistreptococcic activity. They thought as the result of their work that the material was sebaceous in nature and called it "lactosebum". Their analysis showed that the difference in susceptibility to artificially induced Str. agalactiae infection could be related to the fatty acid content of the material. The antistreptococcic substance was isolated in the non-esterified fatty acid fractions and there was also a significant difference in the amount of esterified

fatty acids in keratin from susceptible and resistant quarters. Another group of workers produced results markedly different from those of Adams and Rickard. They (Treece, Morse and Shah, 1964) found a lipid content of eight per cent whereas Adams et al.had found 90 per cent. Their analysis indicated that the material was a protein like substance which was not of milk origin. A later report (Treece and Morse 1965) showed that the lipid fraction also was not derived from milk but neither was it typical of skin lipids. They did not find a relationship between fatty acid composition and resistance to streptococcal mastitis, (Treece, Morse and Levy, 1966). In this, as in many aspects of mastitis there is directly conflicting evidence on the role teat canal keratin has in the initiation of Str.agalactiae infection of the mammary gland. Nevertheless some bacteria do colonise the teat canal (Murphy and Stuart, 1953; Newbould and Neave, 1965; Beech and Forbes, 1965).often for considerable periods without the milk secreting tissue becoming infected. From this it can be inferred that in some quarters the teat canal environment will maintain bacteria and in consequence it is possible they may grow through the canal to reach milk in the teat from where invasion of the milk secreting tissue may occur.

The dry period between lactations is the time when observations on bacterial growth through the canal, unassisted by other factors associated with milking, should be possible. Neave, Dodd and

Henriques (1950) found a high incidence of new infections in teats within three weeks of the last milking of which 52 per cent disappeared before the cow calved again. The predominant infections were "green" streptococci, i.e. Streptococcus uberis, Streptococcus dysgalactiae, Streptococci of groups D and E and other unidentified aesculin splitting streptococci. During three lactations of the herd these streptococci did not predominate during lactation, a fact which the authors thought connected with marked changes in the internal environment of the udder during the dry period. Of the dry period infections which persisted more than half were staphylococci. Dry period infections were determined by expressing secretion from the teats and if two successive samples contained the same bacterium or if one contained large numbers of bacteria they were judged to be from a non-clinical intramammary infection. However, subsequent work (Beech and Forbes 1965) has shown that if the infection was localised in the teat canal the secretion would certainly be contaminated during collection and satisfy the criteria ~~Oliver et al~~ Neave, Dodd and Henriques (1950) used. In the experience of the author quarters commonly produce milk infected with "green" streptococci immediately following calving but very few of the infections are of intramammary origin and are localised in the teat canal. This has been confirmed by collection of milk by syringe from the teat sinus. Oliver, Dodd and Neave (1956a) conducted an experiment to determine if dry quarters that are sampled in the early dry period are more prone to infection

than those which are not, and at the same time to measure the importance of the "so-called natural" seal. They concluded that if natural sealing of the teat orifice or teat sinus takes place it is of minor importance in preventing infection in the early dry period, a statement which infers either that the infections were in fact teat canal infections or that the bacteria grew through the teat canal seal to cause intra-mammary infections.

Neave and Oliver (1962) contaminated teats with S. aureus at the end of lactation and found that most of the teat apices that had been heavily contaminated harboured large numbers of the bacterium three weeks later when compared to other teats. There was a highly significant ($P<0.001$) relationship between large numbers of the bacterium at the teat apex and infection of the quarter, meaning an intra-mammary infection. However, as their criteria for recognising such an infection was large numbers of bacteria in two successive milk samples it is again possible that these bacteria were derived from the teat canal as it is known that swabbing the teat apex with alcohol is insufficient to kill bacteria within the teat canal, (Beech ~~et al~~ and Forbes 1965). The persistence of S.aureus on the teat apex could not be related to teat erosion. They pointed out that heavy contamination of the teat ducts occurred in several teats exposed to small numbers of bacteria or teats used as controls, and one control quarter was later judged to have an intramammary infection.

These investigations on the bacteriology of the dry period shows that new infections occur during the period, but does not satisfactorily

establish whether the infections are confined to the teat canal or reached the interior of the teat. Even when clinical mastitis ensues early in the following lactation it is not definitely known when penetration into the gland takes place.

The evidence available confirms that bacteria colonise in the teat canal but it is not confirmed ~~if~~ that they can grow through the canal to reach the sinus. The prolonged periods during which infections were localised in the teat canal reported by Forbes ~~et al~~ and Hebert (1968) suggests that when infection of the gland did occur the bacteria at least had the opportunity to grow through the canal, but until methods of determining the exact site ~~that~~ where teat canal infections grow and their fate and progression it is impossible to confirm absolutely.

Part II (2)

D. Capillary action in the teat canal

Some of the earliest workers in mastitis considered that bacteria passed through the teat canal by capillary action. Franck (1875) thought that infecting bacteria vegetated in a drop of milk left on the tip of the teat after milking from which they were carried up through the teat canal by capillary action. On the other hand Bang (1888) was unable to produce mastitis by rubbing mastitis streptococci from an agar culture into a drop of milk on the tip of the teat, or by dipping the tip of the teat in a culture of a mastitis staphylococcus. Kitt (1921) suspected at times the capillary crevices in the walls of the teat canal are moist with milk and that when the cistern is full a drop of milk may hang from the teat orifice and assumed that rapidly multiplying, motile organisms could penetrate and pass through the teat canal. Pounden and Grossman (1950) observed that when desquamated cells (i.e.keratin) were removed from teat canals small amounts of milk were contained between the longitudinal folds, whereas small traces or no milk was present between the folds when appreciable amounts of cellular debris were present. They thought this was due to milk repellent properties of the material and considered that it prevented a pathway for growth of bacteria through the canal. Christiansen and Neilson (1934) tied rubber bags filled with cultures of Str. agalactiae onto teats for up to 33 days and were unable to

produce mastitis when the teat canals were intact and not traumatised. The failure of this and similar experiments to cause mastitis is supported by the valid observation of Heidrich and Renk (1967) that "contact of the teat orifice with organisms capable of causing mastitis does not necessarily lead to persistent infection of the udder for in spite of continual intimate association of the teat end with coliform organisms, they are rarely demonstrated in the udder". This observation and other work makes it unlikely that in normal conditions passage of bacteria through the teat canal by capillary action is common. It has, however, been suggested (Oliver, Dodd and Neave, 1956) that when internal pressure in the udder is high it may be sufficient to open the teat orifice allowing milk to escape. This would cause a continuous film of milk from the teat sinus through the teat canal to the exterior of the teat, from where bacteria would have access into the teat sinus. They concluded this from their work comparing new infection rates in the dry period after abrupt or intermittent cessation of milking at the end of lactation. The results showed the infection rate increased with higher yields at drying off. A similar conclusion of the role of high intramammary pressures was reached by Newbould and Neave (1965) who showed that more quarters became infected after S. aureus was inoculated into the teat canals if the first milking after inoculation was missed, than in quarters which were milked normally.

Conclusions

It is evident after reviewing the literature which relates to the teat canal and its function as a barrier to bacterial penetration into the milk and milk secreting tissues, that while there is evidence that it has such a function the mechanism by which it achieves this is not understood. The factors which have a bearing on the efficiency of the canal have been examined without any positive conclusions being reached. The patency of the teat canal is purely one of degree as obviously all canals are patent enough to allow the expulsion of milk and it is evident that if the necessary physical conditions are present, the aperture of the canal presents a huge orifice through which bacteria may pass. "Patent" canals are more dilatable than others and it is possible that if patency does predispose to mastitis, it is due to variation in the functioning of the canal walls, rather than a simple manifestation of a large bore in the canal. This has been discussed in relation to existing teat canal infections and the stresses imposed on the teat canal during milking. If ~~the~~ physical conditions arise whereby bacteria are sucked through the teat canal then it is very unlikely ~~if~~ that the patency of the canal is important. Witzel and McDonald (1965) have shown a residual vacuum in the teat sinus after overmilking and Nyhan and Cowhig (1967) suggest that a fluctuating vacuum during milking might cause momentarily a lower pressure in the teat sinus than at the apex of the teat, conditions in which bacteria

may enter. Further experiments need to be done to examine these possibilities. There is some evidence that a column of milk in the teat canals of cows with high intramammary milk pressures may assist the passage of bacteria into the glands.

However, although these abnormal conditions may facilitate the entry of bacteria, and even allowing for the fact that an estimated 80 per cent of milking machines in Britain are faulty, "normal" cows milked under hygienic conditions by machines used according to specification do develop intramammary infections and mastitis. Until it is known how bacteria reach the mammary gland the methods chosen as preventive measures are subjective and directed at controlling the numbers of bacteria capable of infecting the gland. The incidence of mastitis cases caused by bacteria capable of growing on skin suggests that the skin flora is the main reservoir of infection, but until it is known how they are transported into the udder a level of mastitis in dairy herds is inevitable.

Part II (3)

The bacteria commonly present as intra mammary infections in cattle

The mammary gland is frequently infected with bacteria, some of which are pathogenic resulting in varying degrees of inflammation in the milk secreting tissue. It is natural that the types commonly associated with clinical mastitis have been investigated most thoroughly: other bacteria have been largely ignored, being dismissed as commensal organisms of no significance. The mammary gland may and does harbour bacteria without showing any clinical symptoms. Nevertheless the gland does not have a natural bacterial flora and if bacteria are present, they have infected the gland at some stage during the life of the animal. Whether or not they are sufficiently pathogenic to cause apparent disease is probably unrelated to the facility with which they originally invade the gland. The review which follows analyses the incidence with which various bacteria are present in the udder and also their epidemiology in the environment of the animal and their pathogenicity when present in the gland.

Renk (1967) has reported that since the discovery of Streptococcus agalactiae in 1884, approximately 55 species of bacteria have been demonstrated as causing mastitis. Of these four species cause nearly all cases of clinical mastitis, namely Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis.

For many years Str. agalactiae was the only bacterium to be considered to be important in the aetiology of mastitis. S. aureus was included with other "commensal" bacteria. Thus, Hastings and Beach (1937) considered that mastitis in the absence of Str. agalactiae to be non-specific. Peterson, Hastings & Hadley (1938) described a similar condition although diphtheroids, micrococci and staphylococci were present in the milk, but no importance was attached to them and the presence of a virus was postulated for the aetiology of the condition. It is now realised that such bacteria may cause mastitis, particularly sub-clinical mastitis determined by laboratory tests (McEwan and Cooper 1947, Galli, Gola and Ruffo, 1967, Forbes and Ebert, 1968). However, because of their relatively low pathogenicity, information of their incidence as intramammary infections is restricted to a few reports on the total microflora of the udders of cows in particular herds. The results suggest however that these bacteria are fairly common in mammary glands and a short review of their pathogenicity and epidemiology is included with that of the common recognised pathogens.

Staphylococcus aureus

The bacterium is a common pathogen in nature and since the introduction of antibiotic therapy in the treatment of mastitis it has replaced Str. agalactiae as the most important organism in the aetiology of mastitis.

Its characters, especially its typability by bacteriophages has enabled its epidemiology to be studed in cows and their environment. Spencer and Lasmanis (1952) realised that the development of adequate control measures for staphylococcal mastitis depends on basic information concerning its mode of transmission and its pathogenesis. To gain such information they examined the side of each of the four teats of cows, the hair on the anterior surface of the leg, the vulva and the floor of the stall beneath the cows udder. They found numerous micrococci which would grow in salt agar and ferment mannitol on the skin of the teats but the majority were non-haemolytic and coagulase negative. They did however often find cows with haemolytic micrococci (S. aureus) on the skin of the teats when none were present in the milk. They concluded that the principal extramammary reservoirs for coagulase producing haemolytic micrococci were the skin of the teats and the teat cups of the milking machine. Presumably bacteria on the latter must have been derived from teat skin or infected milk. On the other hand, Schalm and Woods (1953) concluded that the source of pathogenic micrococci for invasion of the bovine mammary gland is primarily other glands shedding the organism. Garvie, Higgs and Neave (1961) isolated

two unusual varieties of S. aureus from swabs of cows teats and Sharpe, Neave and Reiter (1962) found that S. aureus may occur at eroded teat apices and persist for several months without necessarily causing an intramammary infection. Davidson (1961a) reported observations on pathogenic staphylococci in a dairy herd over a six year period. He examined the skin of various parts of the cows and all external orifices and the ecology of each mastitis producing strain was determined by use of phage typing. He found that different sites of the body differed in their importance as sources of infection. The most important source was the udder itself, it being found that pathogenic staphylococci commonly multiply on the udder surface as well as inside the udder. Davidson concluded from a quantitative analysis, that most infections of other sites were derived from the udder.

However by analysing the progression of infections in the herd it is obvious that the skin of the udder is of great importance in the dissemination of the bacteria. He observed a marked difference between strains of S. aureus in their ability to multiply elsewhere than the milk secreting tissue of the udder. The evidence for this was provided by the observation that many of the strains were present in the herd for some weeks or months before they were excreted in the milk. Relatively few strains became widespread in the herd or persisted for long periods but every strain which did persist for more than a few months produced mastitis sooner or later. Thus it appears that the ability of the

bacteria to survive on the skin is associated with their ability to cause mastitis. Some strains, for example strain 1, disappeared spontaneously from the herd without ever being isolated from any sites other than the skin of the udder and teats. On the other hand Strain 29 survived in the herd on the skin of animals, particularly the skin of the udder and teats for approximately 18 months before it was excreted in milk. Strain 2 was introduced into the herd by a donor cow. This cow had one quarter which excreted milk infected with the staphylococcus. This quarter was treated before the cow was introduced into the herd and during her stay in the herd her milk was never again infected. The same strain persisted on the teats of this cow however and from this site it spread rapidly to other cows, soon becoming the most common strain found on the teat skins of the herd. It was first excreted in milk approximately four months after the donor cow's introduction into the herd and after one year caused its first case of mastitis. As the strain persisted on the teat skin it is possible that spread originally occurred by the transfer of machines at milking time. Davidson did state however that the presence of large numbers of organisms in any site almost certainly resulted in the recovery of smaller numbers from many sites on the same cow and other cows. He attributed this to mechanical transfer during milking, grooming and social contact between cows. The last factor he thought responsible for the presence of a strain of staphylococcus on four pairs of heifer twins. The strain was identical to that carried by

some adult cattle with which the heifers had been at pasture before their introduction into the milking herd. Considering the skin is such a reservoir of infection transfer of the bacterium between animals during normal cow management procedures may be an important factor in the spread of pathogenic bacteria.

Cullen and Hebert (1967) reported that S. aureus teat skin infections occurred in more cows than either teat canal or milk infections. Elliott (1965) isolated S. aureus from the vagina, rectum and nose of dairy cows but there was no relationship between the infection being present in these areas and the cow being mastitic. He did report that one cow with an abrasion of the stifle joint was invaded by S. aureus which spread over the body and was resistant to penicillin therapy. Markham and Markham (1966) reported a 12 per cent incidence of nasal carriers of S. aureus in cattle.

S.aureus is therefore a very common bacterium on the body surface of cows, especially on the udder and teat skin. Davidson's work strongly suggests that the bacterium's ability to survive on skin is eventually associated with an increased likelihood of the bacterium causing mastitis.

Coagulas Negative Micrococcaceae

In the literature on mastitis "micrococcci" are often referred to but their identity is seldom satisfactorily established. It can be assumed that they are Micrococcaceae other than S. aureus. This however also needs qualifying for the most important distinguishing character of S. aureus is its ability to coagulate plasma. This character has a high correlation with the production of alpha or beta-haemolysins in strains isolated from bovine sources, and has led to the common practice of simply differentiating haemolytic staphylococci. This is legitimate inasmuch as alpha or alpha-beta-haemolytic strains are almost invariably strains of S. aureus but non-haemolytic strains may also produce coagulase. It is the latter character which is associated with the pathogenicity of the organisms which is paramount in bacteriological examinations relating to mastitis. Schalm and Lasmanis (1957) found coagulase positive strains which did not, or only slightly haemolysed blood and they stressed the importance of using the coagulase test for non-haemolytic cocci in order to detect pathogenic types. Ruffo (1966) found that haemolytic activity failed to identify 11.3 per cent of coagulase positive staphylococci. In the short review which follows coagulase positive staphylococci are described as S. aureus and all other organisms are simply grouped as micrococci. It will be shown later this group is composed of numerous strains of bacteria, some of which are almost certainly more pathogenic than others.

In 1900 Ward slaughtered 19 cows with apparently normal udders and cultured the tissues. He found bacteria throughout the lactiferous ducts of all the glands, nearly all of which he described as micrococci. Harding and Wilson (1913) studied the udder flora of cows and in 1230 milk samples stripped from the udder, 75 per cent contained micrococci. Evans (1916) examined 192 milk samples from 161 cows in 5 herds and examined those bacteria which were present in considerable numbers in the milks. Of the 185 strains she examined, 95 were considered to be S. aureus based on their ability to haemolyse bovine blood, ferment mannite and grow as pigmented colonies. Eight of these 95 cultures which were positive to all these three tests were virulent when inoculated into rabbits and nine other cultures which were positive to only two of the three tests were either slightly virulent or avirulent. It is probable therefore that these were not S. aureus and the proportion of strains considered to be S. aureus may not be as high as the author thought. Nevertheless it is surprising that the significance of micrococci in milk was not fully realised until much later. Murphy (1945) published a report on studies in the genesis of bovine udder infection and mastitis in which he had cultured 1500 milk samples from 195 cows. He found that 77 per cent of all the bacteria were micrococci.

A comprehensive study of the bacterial flora of the udders of the cows in a dairy herd has been reported by McEwan and Cooper, (1947). They related the type of bacteria present in milk samples to mastitis as determined by the presence of clots or an elevation of cell count in the

milk. They showed that although the coagulase negative micrococci were much less injurious to the udder, they could not be dismissed as negligible for 24 per cent of the quarters infected with them had milk showing some rise in cell count. They stressed the significance of this by pointing out that less than 4 per cent of the quarters free from bacterial infection produced milk with a raised cell count. Schalm and Lasmanis (1957) found that micrococci comprised 70 per cent of the types of bacteria isolated from foremilk regardless of the number of lactations the cows had had. The proportion of coagulase positive cocci (S. aureus) increased as lactation age advanced. This was accompanied by a decrease in coagulase negative "saprophytic micrococci" so that the sum of these two groups of micrococci remained the same. Edwards and Jones (1966) isolated coagulase negative staphylococci regularly from 18 cows over a period of 19 weeks. They did not cause a leucocytosis above one million cells per ml. of milk although two other cows infected with S. aureus had cell counts greater than a million per ml. The proportion of cows infected with the two types of bacteria shows the high incidence of coagulase negative staphylococci in some herds. Newbould (1964) reported one quarter which was infected with a "buff non-haemolytic micrococcus" which had cell counts as high as those in another quarter of the same cow infected with an alpha-beta-haemolytic staphylococcus. The coagulase negative cocci are so diverse however that a wide range of pathogenicity probably exists. This was shown by Forbes and Hebert (1968) who reported some intramammary infections which were associated with cell counts

similar to those from uninfected quarters while other infections caused markedly elevated cell counts. Ruffo (1966) examined milk samples from 168 quarters of a herd ... taken at four different occasions and found that 61.9% were infected with Micrococcaceae. The incidence of S. aureus was over twice as high as for S. epidermidis and Micrococcus species.

The presence of an inflammatory state in quarters infected with S. epidermidis was clearly established both by a total and a differential cell count of the milks. Stabenfeldt and Spencer (1966) examined bovine udders which had shed non-haemolytic coagulase negative staphylococci and described lesions in the udder parenchyma which they attributed to the bacterium.

Precise references to coagulase negative micrococci and their pathogenicity are not common in mastitis literature. This reflects the relatively minor importance in mastitis that has been attached to them rather than their incidence in bovine udders. Their importance is now more appreciated and methods to distinguish them being sought. Thus, Sandvik and Brown (1965) and Brown, Sandvik, Scherer and Rose (1967) reported methods attempting to distinguish the bacteria in order that their epidemiology and pathogenicity could be determined. This was because in a newly assembled herd of 20 heifers Staphylococcus epidermidis was found to be the principle cause of udder infections. Edwards ~~et al~~ and Jones (1966) thought that infection with coagulase-negative staphylococci antagonized the colonisation of the udder by a pathogenic coagulase positive strain present in the herd, possibly by an antibiotic

substance inhibitory to S. aureus which they were shown to produce. In order to gain an understanding of the pathogenesis of udder infections, mechanisms such as these will have to be studied and their significance realised in relation to the host.

The Udder Streptococci

Three streptococcal species predominate in mastitis, Str. agalactiae, Str. uberis and Str. dysgalactiae.

Streptococcus agalactiae . Until the common use of penicillin in the treatment of mastitis this bacterium was the predominant cause of clinical mastitis. It is usually considered to be an obligatory intramammary parasite in that it has been thought to be unable to survive very long outside the udder parenchyma. Its pathogenicity once inside the udder is beyond doubt and it is unnecessary to review the considerable literature on this subject here.

If the organism does survive only in the udder then it must spread quickly by direct transmission from cow to cow. However, there are papers which show that this is not the case and which suggest that Str. agalactiae is a common bacterium in the cows' environment, outside the udder. This is of great interest in considering the epidemiology and mode of transmission of the organism.

Bull, Munch-Petersen, Murname, and McLean (1940) for example concluded after an intensive study of a newly established herd, that Str. agalactiae does not have a totally different relationship to the host than do the other streptococci, micrococci or staphylococci commonly found in milk, all of which appeared to be commensal and parasitic in turn. They also showed that a herd, the members of which had no possible contact with a case of mastitis since calfhood still carried Str. agalactiae. The organism could not have been carried in an udder

that had ever functioned but nevertheless it must somehow have been carried from the calves original environment. When the heifers first calved Str. agalactiae was isolated from 16 of 119 functional quarters in the first week of lactation. Their results showed that the infected udder is not the only reservoir of the organism in the dairy cow.

A report on the modes of spread of Str. agalactiae in dairy herds (Imp. Bur. An. Hlth. 1944) showed that some animals were persistent skin carriers without infection of the teat canal or udder. This was frequently associated with the presence on the teats of abrasions or sores but the organism was occasionally isolated in large numbers from teats with apparently normal skin. They also reported that Str. agalactiae could be isolated from milker's hands and on objects in the cowshed, results confirmed by Chodkowski and Lancaster (1949) but concluded that the principle reservoirs were the surface of the udder and the mammary parenchyma.

The possibility that the udder surface acts as a reservoir for infection is perhaps not consistent with the fact that complete elimination of this bacterium from dairy herds has been achieved by the use of penicillin administered simultaneously into all infected mammary glands.

It is known, however, that herds from which the organism had not been isolated for several years occasionally do have recrudescence of the disease, often without any animals being introduced from other herds (Wilson 1968). It is possible of course that the infection may have been carried into the herd by other vectors but it is equally

possible that Str. agalactiae may have persisted on the skin of the cows.

Streptococcus uberis . Sweeney (1964) investigated the epidemiology of Str. uberis throughout a lactation. He found that the frequency of isolation from the udder surface was always high and that infection at this site was sometimes independent of intramammary infections although there was a definite relationship between the bacterial populations of the two. His evidence suggested that the udder surface was the most important reservoir for Str. uberis as 73 per cent of isolations from 12 body sites were from the udder surface. This preponderance on the udder surface together with the observation that they were sometimes independent of but always coincident with infection in the milk suggested to him that infections of the udder parenchyma were secondary to skin infection. His results of the epidemiology of the organism agreed closely with the seasonal variation in incidence reported by Hughes (1960) and Berger and Francis (1951). These findings were largely confirmed by Cullen (1966a). He agreed that the skin was the most important reservoir of infection but by using special selective cultural methods he was able to show that different skin sites had different isolation rates, the belly and lips being most heavily infected. The skin of the teats was a comparatively unfavourable site although he too agreed that milk infections were secondary to skin infections. In a later paper (Cullen and Hebert 1967) Str. uberis was isolated

much more frequently from milk and teat skin than teat canals.

Streptococcus dysgalactiae. Str. dysgalactiae is a common cause of mastitis and yet little work has been done to investigate its epidemiology. Francis (1941) isolated the organism from bovine tonsils and vaginas. Ingalls and Johnson (1947) and Neave, Sloan and Mattrick (1944) isolated it from teat skins, the latter workers from a herd of cows which had not previously had mastitis caused by the bacterium. Taylor (1949) showed that although Str. dysgalactiae disappeared quickly from a wound that had been experimentally infected it was able to survive for some time on skin after experimental infection. He concluded from his experiments that Str. dysgalactiae and Str. uberis are not strict parasites and should be regarded as saprophytes which may become parasitic. This was supported by Solberg (1968) who isolated Str. dysgalactiae from nine different pathological conditions in horses, cows and pigs. He suggested that the organism is widely distributed and is more important than it has previously been accredited.

The Udder Corynebacteria

The presence of Gram-positive bacilli in milk was first reported by Evans 1915, although by then the bacteriology of milk had been intensively studied. The fact that until then they had been overlooked was because of their slow growth on the media normally used. During incubation periods used for the demonstration of cocci these bacteria were barely visible and needed several days growth on serum agar to be seen. Evans originally reported the bacterium to be Bacillus abortus which was amended to B. abortus var. lipolyticus (Evans 1916) and then to Bacterium lipolyticus (Evans 1918(a)). She showed (Evans 1916) when her cultural methods were fully developed that in 37 milk samples from one herd the organism was present in 19 (51.4 per cent). Bendixen (1932) isolated a similar organism from clinically normal udders but also reported that it could be found in the secretion of clinical cases of mastitis although it often occurred together with other bacteria.
He examined 77 cultures and found 71 of these to be similar and thought to be identical to B. lipolyticus as described by Evans. Jayne-Williams and Skerman (1966) discuss the way which this bacterium came to be called Corynebacterium bovis in Bergey's Manual. Abd-el-Malek and Gibson (1952) thought there was a difference between C. bovis and the Evans strain, a difference which Jayne-Williams and Skerman. found confined to the urease reaction, Evans' strain being negative.

The pathogenicity of corynebacteria when present as intramammary infections was confirmed by McEwan and Cooper (1947). They found that the udder corynebacteria caused an elevated cell count and in order of importance followed Str. agalactiae and S. aureus only, particularly when considering the less severe reactions. In the aetiology of sub-clinical mastitis corynebacteria were of similar importance to Str. agalactiae. They concluded in fact, that any bacterial infection of the udder which is not entirely transient in character may be the cause of an udder reaction. They also found a very high incidence of quarters infected with corynebacteria. In quarters free of other bacterial infections 67, 88 and 97 per cent were infected with the bacterium at the first, second, third and subsequent lactations, and it was the most infective bacterial species in the herd they examined. Despite this work C. bovis is still considered by many to be a commensal organism.

The insignificance attached to C. bovis as an intramammary pathogen was corrected by Cobb and Walley as recently as 1962. In 2,772 milk samples, C. bovis was isolated in pure cultures in 31.2 per cent, and 3.4 per cent contained clots. It was shown that intramammary inoculation could cause clinical mastitis and further, that in one herd fine clots in milks could only be associated with the presence of C. bovis. From this they stressed that C. bovis should not be dismissed and ignored as a commensal of the bovine udder. Similarly Wilson and Brookbanks (1967) suspected that some cases of subclinical mastitis were associated with

C. bovis. Bourland, Marshall, Hindery and Turner (1967) reported that 90 per cent of the quarters in an experimental herd were infected with C. bovis during a nine month period.

The importance of C. bovis in the udder may not only be its own pathogenicity but that it induces a low grade inflammatory response in the udder and this may confer a protection to subsequent invasion by other bacteria. Newbould and Neave (1965) found a significant difference in the recovery rate of S. aureus ten minutes after being infused into udders infected with corynebacteria when compared to uninfected glands. The leucocytes were twice as high in the former and this the authors suspected affected the recovery of staphylococci rather than the presence of corynebacteria.

Wilson (1958a) reported a species of Corynebacterium (C. ulcerans) to be causing mastitis and since then the incidence has increased. Higgs, Smith, Cleverly and Neave (1967) reported that the infection was found in 13 out of 36 dairy herds. In 12 herds the incidence was low but in one 48.9 per cent of the udders were infected and of these 71 per cent of the quarters were infected. They further showed by the results of the Whiteside test and cell count that the infections were as severe as those caused by pathogenic staphylococci and streptococci.

The extramammary habitat of C. bovis has not been defined. However it is known that corynebacteria are very common resident bacteria of human skin and sorting them into characterised species is in the opinion of Kligman (1965) totally impossible at present. Newbould (1965) observed that C. bovis colonies grow on blood agar plates sown with milk, usually where the milk fat is deposited. He was unable to demonstrate this bacterium in milk soaked swabs from the teats and teat orifices of cows known to be shedding the organism in their milk. He showed that C. bovis had a fatty acid requirement which was satisfied by Tween 80 and concluded that the organism was similar to corynebacteria isolated from human skin.

Conclusions

This brief review of the epidemiology of the bacteria commonly present in milk collected aseptically from the cow has shown that the skin of the cow has or can have these bacteria as part of a resident flora. It is impossible to know by skin sampling techniques whether the bacteria isolated are resident inhabitants of the skin or contaminants present on it at the time of sampling. The only guide is the frequency with which particular species can be isolated in large numbers from the same site. Cullen and Hebert (1967) showed that non-haemolytic staphylococci were the most common bacteria of the teat skin throughout a lactation period, whereas C. bovis was the most common bacterium in milk. It is right that the bacteria which infect the mammary gland and then cause mastitis should have received most attention but it is difficult to find evidence that their parasitism is unique as compared to other bacteria sharing a similar environment. This is best illustrated by considering their "infectivity", i.e. the ease with which they spread and establish persistent infections. Bull et al (1940) found in a herd in which corynebacteria were significantly absent that micrococci had the greatest infectivity and produced the most permanent infections of the udder although having little pathogenicity. The micrococci were followed by staphylococci (i.e. S. aureus) and then Str. agalactiae. Streptococci other than group B were the least infective of the more common bacteria of the cows' udder. In a herd in which corynebacteria were present McEwan ~~et al~~ and Cooper (1947)

found that bacteria arranged in descending degrees of infectivity were as follows: 1) The udder corynebacteria 2) the coagulase positive haemolytic staphylococci 3) Str. agalactiae. 4) non-haemolytic micrococci 5) coagulase negative haemolytic staphylococci 6) Str. uberis and Str. dysgalactiae.

The presence of corynebacteria in one of these herds was the most obvious difference between them but the similarity of infectivity is striking. The epidemiology and extra-mammary environment of the corynebacteria has not been studied but it is known that corynebacteria are very frequent inhabitants of human skin.

It seems probable that the incidence of mastitis is dependent upon the chance encounter of bacteria with the intramammary environment. The subsequent pathogenesis of the disease is further dependent on: i) the presence or absence of prior or existing infections ii) the virulence of the invading bacterium iii) the susceptibility of the host animal.

It seems an impossible task to selectively eliminate pathogenic bacteria from the cows' environment and this stresses the importance of studying the mechanisms by which infection establishes within the udder in order to ascertain if means can be found to prevent it happening. This would almost certainly mean the elimination of "commensal" as well as pathogenic bacteria from the mammary gland, because the incidence of various types of bacteria commonly present as intra-mammary infections is probably proportional to their incidence and weight of infection on the skin of

the host animal. Certainly the similarity of the resident bacterial flora of the skin and of the udder parenchyma and in contrast to the contaminants to which the bovine skin is exposed, cannot be ignored as a factor in the development of intramammary infections.

Part II (4)

Skin bacteria and factors affecting skin colonisation with special reference to the teat canal

It has been ~~shown~~ noted in a previous section that the normal bacteria of/skin human are Gram-positive Micrococcoceae and corynebacteria. Although the latter have not been adequately demonstrated on bovine teat skin it has been argued that its flora is similar to that reported on human skin. It has also been pointed out that these bacteria are those most commonly present in the mammary gland. Bovine skin is obviously exposed to numerous bacterial species in the normal environment of the animal and most of these types fail to establish on the skin. To qualify as a resident bacterium of skin, the organism must be able to multiply there and not merely survive on it. When the site to be sampled is as contaminated as bovine skin usually is, the test for this faculty of persistence largely depends on the repeated recovery of considerable numbers of the organism from a majority of the population examined. The fact that not all bacteria are capable of colonising skin is probably due to their incapacity to adapt to the skin environment since other bacteria can form a resident flora on it. The factors which influence the skin environment in relation to bacteria have mostly been investigated on human skin and recourse must be made to this work. The findings will be compared with published work on bovine skin, particularly in its relation to bacteria causing mastitis.

Factors affecting skin colonisation by bacteria

It has long been realised that bacteria placed on human skin in fluid suspension quickly disappear. Schiemann and Landau (1919) incriminated the acidity of the skin but later Norton and Novy (1931 and

1932) reported that the rapid disappearance of bacteria applied to the skin coincided with the drying of the suspension and that if the skin is kept moist little reduction takes place.

They also showed that if a bacterial suspension is allowed to dry on glass instead of skin then the rate of disappearance is almost as quick. They concluded from their experiments that the skin does not have specific bactericidal properties and the effect is primarily due to desiccation. Cornbleet (1932) showed that when moist skin folds are exposed and allowed to dry their self sterilising power is evident, whereas when juxtaposed and moist, sterilization was negligible after an hour. Burtenshaw (1938) spread a culture of a beta-haemolytic streptococcus on his fingers and measured the reduction at 2-4 minutes and 60-90 minutes after inoculation. From this work he concluded that the self disinfecting power of the skin functions after killing by drying is complete, and it is a much slower phenomenon. In 1942 he showed that skin extracts containing long-chain fatty acids killed alpha - and beta-haemolytic streptococci and Corynebacterium diptheriae; were variably effective against staphylococci and ineffective against Gram negative bacteria in vitro. Rebell, Pillsbury, de St. Phalle and Ginsburg (1950) investigated the rapid disappearance of bacteria placed on normal skin. They used Bacterium coli, 2 strains of S. aureus, a normal skin coagulase negative staphylococcus and a group G. streptococcus as test organisms. Their conclusion was that there was no evidence warranting the assumption of an antibacterial factor other than desiccation.

They also showed that the coagulase negative skin-inhabiting staphylococcus was no more resistant than S. aureus to auto-disinfection. In addition they found that streptococci after the initial decrease during the drying period showed no further reduction in count. This was in contrast to Burtenshaw's work whose results may have been due to the initial sampling being done before drying of the suspension was completed.

Ricketts, Squire and Topley (1951) investigated the sterilising power of skin under dressings which prevented or permitted natural drying. Streptococcus pyogenes disappeared within one day and S. aureus within three days whether or not drying was prevented or permitted. The bactericidal factors were shown to be fatty acids as the sensitivity of the test bacteria to these substances was shown to run parallel with their rate of disappearance on moist or dry skin. The authors concluded that for Str. pyogenes the major factor was the action of unsaturated fatty acids, probably oleic acid, for S. aureus both unsaturated fatty acids and drying had a demonstrable influence and the Gram-negative bacteria were susceptible to drying only.

The fact that water is critically important in regulating the total number of bacteria on the skin was confirmed by Marples (1965). He applied various dressings to the forearms of volunteers and these were designed to achieve various degrees of hydration. By these means he studied ecological and environmental modifications on the natural microflora of the skin. The methods produced a great increase in the

numbers of organisms per unit area and the difference was proportional to the degree of hydration produced. The proportionate composition of the flora changed sequentially. At first the numbers of staphylococci increased giving way to lipophilic organisms, a few Gram negative rods and diphtheroids. The diphtheroids later predominated in association with a constant small proportion of the other bacteria. The author states that all these bacteria are normally found on some areas of skin although on the normal forearm Gram negative rods are undetectable but after hydration these become significant. Similarly Blank and Dawes (1958) concluded that the low moisture content of skin alone plays an important part in preventing the multiplication of organisms because an increase in moisture content of the stratum corneum without other changes permitted the organisms to multiply.

Kligman (1965) questioned the opinion that the skin has self disinfecting powers and that it kills organisms alien or dangerous to it. He questioned the evidence upon which this is based, pointing out that although organisms, even normal skin bacteria, when applied to the surface disappear in seconds or minutes, this also happens on glass and leather. Desiccation is responsible and this cannot be considered a specific biologic defence. Rather than claim that the skin kills all the organisms which are not normally found on it, he considered it more probable that the normal flora predominates because it is best adapted to the habitat provided by the skin.

He also discussed the localisation of the resident skin bacteria and considered that there is convincing evidence that bacterial colonisation is limited to the superficial subsurface zones in the uppermost three to five cell layers of the stratum corneum. Groups of horny cells in these layers separate and the layer begins to crack and spaces appear. Into these crevices skin lipids and products of keratinization seep and when a bacterium finds the niche a microcolony is formed. In another paper (Kligman 1963) he showed that this material is the substrate upon which bacteria survive. The fatty acids present in sebum, are not responsible for killing the bacteria but in fact are produced by them. Nicolaides and Wells (1957) showed that skin lipids in sterile cysts contained no free fatty acids and are present in bound esterified form. Bacteria split the fat to liberate free fatty acids. Pillsbury and Kligman (1954) in fact showed that the richest sebaceous areas on the human skin supported the largest number of resident bacteria. Kligman (1963) also provided evidence that sebum physically protects organisms, in that it diminishes the effects of desiccation.

The factors affecting bacterial colonisation of skin have already been summarised and it is now necessary to relate these factors to the environment offered by the skin of the bovine teat canal.

Firstly it is known that the degree of hydration of the skin is important for bacterial colonisation. Cornbleet (1932) showed that skin which is juxtaposed and moist maintained viable bacteria and that

sterilisation was negligible after one hour. The teat canal being lined completely by skin and whose surfaces are normally held in apposition by the spincter offers just such an environment. In addition the only times the surfaces are not apposed milk is flowing across them. Consequently the drying effect in such an environment is extremely small. Marples (1965) states that the horny layer of skin holds about 10 per cent of its dry weight of water. Prolonged immersion in water of the isolated horny layer enables it to take up water equivalent to 250 to 300 per cent of its dry weight. In the teat canal desquamated cells never reach the horny stage as on exposed skin areas and possibly in combination with sebum form the malleable substance described as teat canal keratin. The degree of hydration of this material is obviously higher than its counterpart on exposed skin surfaces.

The amount of sebum present in teat canal keratin has been the subject of two totally conflicting reports. (Adam ~~et al~~ and Rickard 1963, Treece et al. 1966). Morse and Levy The former authors found the lipid content of keratin to be 90.9 per cent whereas the latter found it 36.3 per cent. This difference prompted Treece et al to contest the sebaceous nature of the material and they thought it more keritinaceous in character. Adams ~~et al~~ and Rickard (1961) and Helmboldt ~~et al~~ Sunghera and Plastridge (1953) demonstrated cells in the teat canal whose activity was thought to be the production of sebum. Whatever the lipid content of keratin, it is probable that it supplies nutrients to the bacteria growing in it, rather than being bactericidal.

Adams et al (1963) did find that keratin from quarters resistant to artificial challenge of Str. agalactiae had a greater antistreptococcal activity than that from susceptible quarters and this they attributed to a significantly larger amount of esterified fatty acids. Treece et al (1966) on the other hand found no relationship to fatty acid composition and resistance to mastitis. However, in view of the work of Ricketts ~~et al~~ Squire and Topley (1951) it may be that the fatty acids do exercise an inhibitory effect upon streptococci. Staphylococci, however, may be partially responsible for the production of fatty acids from sebum and lipophilic corynebacteria have a fatty acid requirement essential for growth which is satisfied by constituents present in sebum (Pollack, Wainwright and Manson, 1949, Newbould, 1965). In addition to the effects of bacterial metabolism on skin, Scheimann et al (1960) suspected that the presence of lipolytic esterases of cellular origin were also important in lipolysis on the skin. Nevertheless the fact that some skin bacteria produce free fatty acids as a by-product may be a factor by which other bacteria are unable to colonise the skin. Kligman (1965) thought there to be little doubt that the coagulase negative staphylococci resident on skin exert a restraining influence on colonisation by other organisms to which the skin is inevitably exposed. The production of fatty acids by staphylococci may be a factor in this antagonism. Pophristov and Todorov (1965) demonstrated that white non-haemolytic staphylococci have properties in vitro which show pronounced antagonism to a wide spectrum of pathogenic and non-pathogenic test bacteria.

In addition to lipids keratin also contains basic proteins as shown by Hibbitt and Cole (1968). They extracted proteins which were shown to markedly inhibit 2 strains of S. aureus and Str. agalactiae, the staphylococci being the more susceptible. Thus, extracts from teat canal keratin have been shown to be inhibitory to the two most important pathogens causing clinical mastitis. Nevertheless, there is considerable evidence to suggest that bacteria survive on the skin for long periods and in addition the anatomy of the teat renders the epidermis of the teat canal a peculiarly suitable environment. Moreover Sharpe, ~~et al~~ Neave and Reiter (1962) Beech ~~et al~~ and Forbes (1965) and Forbes ~~et al~~ and Hebert (1968) have shown that the common mastitis pathogens can live in and around the teat canal for extended periods. The in vitro effects shown on bacteria by extracts of keratin may be factors which influence the susceptibility of the tissue to infection in vivo but it is likely that overall the teat canal environment is suitable for bacteria, particularly those able to colonise normal skin.

PART II (5)

The pathogenesis of mastitis

Introduction

The word mastitis means an inflammation of the mammary gland parenchyma. Many factors can cause the inflammation, such as trauma (Petersen 1964), the introduction of any foreign fluid into the gland (Garrison ~~et al~~ and Turner 1936) but the most common and important cause is the presence of bacteria within the udder parenchyma. It is the pathogenesis of bacterial mastitis which is to be considered here.

For infectious mastitis to develop an infective dose of bacteria must penetrate the teat canal and subsequently grow within the gland. Possible mechanisms by which bacteria pass through the teat canal have already been reviewed. Unless the bacteria are derived from another infected gland and the transmission is direct to an uninfected gland the environment offered to the invading bacteria in the gland is totally different from their recent habitat.

If the bacteria survive the period immediately following invasion then the parasitic potential of the organisms can be manifest. Factors which influence the survival of the bacteria are 1) the suitability of the environment within the gland 2) the susceptibility of the host to the infection and 3) the pathogenicity of the bacterium itself.

The suitability of the intramammary environment for bacteria

When bacteria are inoculated into a suitable medium the rate of growth at first is slow, a period described as the lag phase. This is followed by the adaptation of the organisms to the environment and a period of rapid growth, the log phase. It is probable that during the lag phase some cells of the bacterial population die due to a failure to adapt to the new medium. For this reason a certain minimum infective dose must be inoculated in order to ensure the survival of sufficient cells to allow their further multiplication. This phenomenon was well shown in relation to milk as a growth medium by Baumgartner, Kästli, Walser and Meeder (1965) who conducted a series of 28 experiments in which Streptococcus lactis, S. aureus and Escherichia coli were grown in broth or milk from which they were subcultured into fresh sterile milk. Growth of the inoculum from the broth was inhibited for up to 2½ hours. The authors concluded that growth inhibition in fresh milk was due to a phase of adaptation to the growth substrate (i.e. milk) rather than to the existence of specific inhibitors.

Jones and Little (1927) had shown that no significant growth of Str. agalactiae occurs in cows' milk within six hours of its inoculation. However, Murphy and Stuart (1952) while confirming this delay in growth pointed out that after six hours the bacterium was reaching its log phase of growth. They concluded that the inhibition of growth by milk would seldom if ever represent a significant barrier to infection of the gland.

Other workers (Hanssen 1924, McEwen and White 1950, ~~and~~ Auclair and Hirsch 1953) reported milk to have specific inhibitors which were bactericidal to many pathogenic and non-pathogenic bacteria. Jones and Simms (1930), Wilson and Rosenblum (1952a) and Auclair and Hirsch ~~et al~~ (1953) showed the inhibitory effect was due to substances called lactenins.

Lactenin is present in the whey fraction of milk and is inactivated by exposure to a temperature of 80°C or higher. It has been found in the milk of all cows although variations in titres are recorded depending on the time of day relative to milking and the quarter from which the milk was taken. Jones and Little (1952) showed its presence in milk appeared to be due to formation in the mammary gland itself rather than excretion through the gland since it was not present in the blood of cows whose milk contained it. However, Wilson and Rosenblum ~~et al~~ (1952b) showed lactenin to be inactive within the udder because the milk is then at a low oxidation-reduction potential at which the substance is inactived.

The susceptibility of the host to infection

Providing an adequate infective dose passes through the teat canal the milk in the gland has no immediate specific inhibitory action. The host however does respond in two ways if the infection causes an inflammation; firstly by the production or liberation of antibodies and secondly, by leucocytes passing into the gland. The liberation and production of humoral and local antibody will be considered first.

A. Antibody in milk

Darbyshire (1964) investigated the multiplication of S. aureus in samples of normal milks and in naturally infected samples from cows with staphylococcal mastitis. The milk from mastitic cows was relatively inhibitory to multiplication. Cell free milk from a cow with an induced traumatic mastitis was shown to have a heat labile humoral component which was bacteriostatic to the staphylococcus. Similarly Jain, Jasper and Carroll (1967) showed that cell free normal milk was nonbactericidal to Aerobacter aerogenes whereas a similar fluid from an udder with an induced traumatic mastitis was bactericidal. The bactericidal factor in the latter was heat labile and the bactericidal effect of unheated mastitic fluid decreased as the time from the induced trauma increased. This and other evidence showed the bactericidal factor to be humoral in origin. The bactericidal activity of cell-free mastitic milk generally coincided with increases in serum albumin and immune globulin fractions of the whey proteins. The leakage of plasma proteins into whey is known to depend on capillary permeability which is increased during

inflammation. Holman,Pattison and Gordon (1952) showed that goats with a very high serum titre to Str. agalactiae had very low titres of agglutinin in their whey. Miller and Heishman (1943) showed that the mammary epithelium offers a very effective barrier to staphylococcal antitoxins passing from blood to milk. Except at the time of parturition (Edwards and Smith 1959) and during mammary gland involution at the end of lactation (Kerr, Pearson and Rankin 1959) antibodies do not pass through the mammary epithelium into milk, even in animals with high serum titres. Darbyshire (1960) showed that irritation of the mammary epithelium was necessary before circulating antibody could pass into the milk. A very mild irritant (10 ml. of saline) introduced into the gland was sufficient to allow antibody to pass. This means that when bacteria invade the gland and start to multiply, until they produce a degree of mastitis, specific antibody cannot affect them. The first stage of infection therefore is independent of the immune status of the host. This is consistent with the work of Howell, Smith, Holman and Pattison (1956), Slanetz,Bartley and Allen (1959) and Darbyshire (1960b) who recorded mild mastitis in vaccinated animals.

There is some evidence that antibody is produced locally within the mammary gland. Campbell, Porter and Petersen (1950) described the transitory appearance of plasma cells in the udder at the time of parturition. More recently Willoughby (1966) demonstrated sites of antibody production in the gland by the use of fluorescent immunohistochemical techniques. Local production of antibody following the

infusion of antigens into the dry mammary glands of pregnant ewes has been claimed by Lascells, Outteridge and MacKenzie (1966). Their results indicated that both local synthesis and active transfer of antibody occurred. They also found evidence of continued local antibody production in the gland throughout a lactation of 8-12 weeks and suggested that the cells responsible for its synthesis were in close proximity to the glandular epithelium. In experimentally induced staphylococcal mastitis Outteridge and Lascells (1966) found a higher antibody titre in the secretion from the infected glands of two ewes than in their plasma. This suggested evidence of local antibody production to the authors. Pierce and Feinstein (1965) found that the immune lactoglobulins in bovine colostrum were qualitatively similar to those in serum. However, there were marked differences between relative concentrations in serum and colostrum. They produced definite evidence for selective transport of serum proteins into the mammary gland which was related to their electrophoretic mobility. While their studies were related to colostrum production it is possible that selective transport continues during lactation to cause higher titres in whey than plasma. It is more common nevertheless for plasma titres to be considerably higher than those in whey.

In summary it is evident that an immunologically susceptible animal has no immediate defence to prevent bacteria multiplying in milk in the mammary gland. There is also strong evidence that until a degree of mastitis has already occurred humoral antibodies in an immune animal do

not reach the intra-alveolar spaces where they may be effective. The hosts' defence mechanisms therefore can act to control an existing infection but, apart from the barrier of the teat canal, can do nothing to prevent its inception. Darbyshire (1962) in a review of immunity in bovine mastitis concluded that humoral antibacterial factors normally present in milk have little influence on the course of a mastitis infection and that their effect is probably overshadowed by the cellular response to infection. This aspect will be considered next.

B. Cells in Milk

It will be shown in the following review of recent literature that cells in milk influence the fate of intramammary infections. Various types of cells have been described in milk. All the types of white cell present in blood have also been demonstrated in milk (Christiansen 1929) in addition to epithelial cells which are presumed to derive from the ducts and acini of the udder parenchyma. The latter were subdivided into six different morphological types by Zlotnik (1947). Because of the time consuming and laborious methods needed to differentiate recognisable cell types, it is usual to use the total numbers of cells in milk as an indication of inflammation. The numbers of cells judged to be significant is rather arbitrary and different workers have very different opinions. In fact the numbers of cells present in normal milk is a matter of much controversy, largely because there is no definite criterion by which to estimate normality.

Different fractions of milk from the same quarter vary in cell content (Paape and Tucker, 1965; Smith and Schultze, 1966, ~~and~~ Beech 1967): there is also a diurnal variation in milk from the same quarter (White and Rattray, 1965, Cullen 1967a, and Smith and Schultze ~~et al~~ 1967) and a variation during the course of lactation (Blackburn 1966, Forbes and Hebert, 1968) and in successive lactations (Waite and Blackburn 1957, Blackburn, 1966). These variations have all been discussed more fully in a review by Cullen (1966b).

It is known that some cells in milk are capable of phagocytosing bacteria (Katsube and Blobel, 1964, Jain and Jasper, 1967, Schalm, Lasmanis and Jain 1967) although destruction of the ingested bacteria does not always follow, (Jain et al 1967, Katsube ~~et al~~ and Blobel 1964, Schalm ~~et al~~ Lasmanis and Jain 1967, Newbould ~~et al~~ and Neave 1965b). Indeed Newbould, Saurasti and Barnum (1966) showed that the presence of leucocytes from bovine milk stimulated the multiplication of mastitis staphylococci in vitro.

~~He~~ Newbould (1967) further showed ~~(Newbould 1967)~~ differences between groups of leucocytes in their competence to ingest staphylococci and in the numbers of staphylococci ingested. Such differences occurred in leucocytes from different quarters of the same cow, a factor which he thought may be due to variations in the permeability of blood vessels and tissues in blood constituents. The leucocytes in these experiments were from milk of glands ~~who~~ which had a mastitis induced with saline injections which is known to alter the permeability of vessels. Jain and Jasper (1967b) showed it increased both the number and percentage of viable cells in milk.

Galli and Guallini (1966) considered milk to be normal when it contains less than 12 per cent polymorphs, suspect when the proportion is between 13 and 19 per cent and infected when over 20 per cent. However, as Waite and Blackburn [illegible], (1957) showed that the proportion of polymorphs increases as the total cell content increases an estimation of the total number of cells in milk is still a valid criterion by which to judge the degree of inflammation in the udder. The relationship between the cell content of milk and the susceptibility of the udder parenchyma to infection and the subsequent pathogenesis will now be considered.

The role of cells in the pathogenesis of mastitis

It has been shown by various workers that the leucocyte response to infection of the mammary gland may control the infection. Murphy and Stuart (1953a) inoculated eleven quarters of four heifers with 35 ± 31 Str. agalactiae cells. Four of the quarters became infected and these were all previously uninfected and at the time of inoculation were producing milk with low cell counts. Acute mastitis followed during which phase the bacterium was eliminated from two of the quarters. The acute symptoms subsided in the other two quarters and were followed by a state of chronic infection. Thus in two of the quarters the inflammatory response was efficient in eliminating the infection. In the same series of experiments two quarters, one being infected with corynebacteria and the other with staphylococci, did not become infected or show any clinical signs after repeated inoculations of Str.agalactiae. The authors suspected this was due to these quarters having a higher cell content, especially considering their previous work (Murphy and Stuart, 1952) which demonstrated that infected milk with a raised cell content had greater inhibitory power for Str. agalactiae in vitro than normal milk.

Schalm and Lasmanis (1963) recorded the spontaneous occurrence of acute coliform mastitis in a herd in which staphylococcal and streptococcal mastitis was successfully controlled and this led them to a study of the effects of a pre-existing leucocytosis upon experimentally induced mastitis. They first showed that approximately ten Escherichia coli ×

could cause an acute mastitis and the bacterium was eliminated during the acute phase of the disease. They then showed that a pre-existing leucocytosis induced by a Seitz filtered S. aureus broth culture injected into the gland 24 hours before or simultaneously with E. coli protected the gland from infection. The cell count rose to 5 million or more cells and this conferred protection in some quarters even when 1000 E. coli were inoculated daily for four days. As a result of their experiments they suggested that it is not desirable to aim for complete elimination of cells in milk as the glands may then be particularly susceptible to mastitis. They subsequently showed (Schalm, Lasmanis and Carroll, (1964a) that the cows were still susceptible to the infection when all evidence of an inflammatory process had disappeared. The cell level in milk which inhibited unrestricted growth of the inoculum was between 200,000 and 500,000 cells per ml. Above this level there was complete inhibition. It is of interest to note that a mild inflammatory reaction due to infection with Str. uberis inhibited multiplication of 500,000 Aerobacter aerogenes whereas another, but normal, quarter of the same cow responded peracutely to an identical inoculum. In another paper (Schalm, Lasmanis and Carroll 1964b) which investigated the pathogenesis of Aerobacter aerogenes mastitis they concluded that phagocytosis appeared to be prominent in clearing the gland of the bacterium and if the inflammatory response subsided too quickly and before all the organisms were cleared from the gland, a recidivation of acute mastitis was probable. Schalm, Carrol and Lasmanis (1964) found that a cell level of 200,000 per ml or greater was associated with a high degree of protection

against the potential of an inoculum of 66 to 218 viable A. aerogenes organisms to produce mastitis. They later demonstrated that a marked hormone induced neutrophilia did not give any protection to experimentally induced A. aerogenes mastitis (Schalm, Lasmanis and Carroll 1965a) nor did the use of a synthetic corticoid (an anti-inflammatory drug) delay or inhibit the infiltration of leucocytes into the mammary gland in response to exposure to the bacterium. (Carroll, Schalm and Lasmanis 1965).

In a later paper Jain, Lasmanis and Schalm (1967) were able to delay the inflammatory response by inducing a leucopenia in the cow prior to inoculation of the mammary gland. This allowed A. aerogenes to multiply to a peak of 90 million per ml of milk which was far higher than in their previous work in cows with normal levels of circulating neutrophils.
The induced leucopenia also had a most interesting effect upon a quarter with a naturally occurring infection of a coagulase negative staphylococcus. The bacterium multiplied rapidly during the leucopenic phase allowing what had been a subclinical mastitis to develop to an acute reaction in 24 hours. Later a massive infiltration of neutrophils caused the staphylococcus to decrease in numbers and eventually to disappear from the gland. The authors pointed out that this admirably demonstrated how a depletion or weakening of the leucocyte barrier may permit a normally innocuous bacterium to multiply unrestrictedly and produce acute disease.

Leucocytic infiltration into milk is also significant against other bacteria. Blobel and Katsube (1964) showed that an experimentally induced

leucocytosis in milk protected 3 of 4 glands inoculated with S. aureus and all 4 glands inoculated with A. aerogenes. The four untreated control quarters in each experiment all became infected although only temporarily with A. aerogenes. Str. agalactiae established in both treated and untreated quarters, the only difference being that multiplication of the organism was delayed in the latter. Ten thousand streptococci were injected into each gland in this experiment. Schalm, Lasmanis and Carroll (1966) showed that the survival of such a dose was dependent upon the rate and magnitude of the cellular response.

Doses of 10,000 to 25,000 streptococci failed to establish infection if an intense and rapid cellular reaction followed the inoculation. If, on the other hand the reaction was delayed the inoculum survived and established a chronic infection. Small doses of 5 to 350 streptococci however established infection in six out of nine normal quarters of cows in their first lactation. The determining factors were the numbers of bacterial cells inoculated and the magnitude of the early cellular response. If this response was delayed 12 to 24 hours the streptococcus established a chronic infection. Once the infection had established the subsequent inflammatory cellular response was usually incapable of eliminating it. In a later paper Schalm ~~et al,~~ Lasmanis and Carroll (1967) inoculated 1,000 colony forming units of Str. agalactiae daily into quarters recovering from sterile inflammation. In three quarters there was a delay of up to 16 days before infection resulted. The authors thought the suppression of the inocula was due to humoral factors since a marked cellular

reaction was not always evident during the course of the inoculations. Klastrup (1956) was able to produce mastitis in cows in their first lactation by inoculation of small numbers of S. aureus. Six or more cells in log phase growth had a 52 per cent probability of inducing infection. His experiments indicated that the smallest doses tended to cause latent or sub-clinical mastitis whereas large inocula caused acute disease of short duration.

Summary

In the introduction to this section it was pointed out that a minimum infective dose was essential to establish an infection. The minimum infective dose for the mammary gland for any bacterium cannot be standard for it has been shown in this review that the susceptibility of the host to intramammary infection is dependent upon the intramammary environment at the time of infection. It has also been shown that a large dose may be eliminated from the gland because it provokes an immediate intense reaction by the host whereas lower doses do not and the infection may therefore establish. Klastrup (1956) showed that six S. aureus cells in log phase growth in milk in vitro could infect a mammary gland. Murphy and Stuart (1953) showed in the case of Str. agalactiae that approximately 30 cells caused mastitis while Schalm and Lasmanis (1963) showed ten E. coli was an infective dose. All these experiments were done with susceptible animals. On the other hand much larger numbers of bacteria can be eliminated if the gland is secreting milk with a high cell count, whether artificially induced or due to an existing low grade infection.

The existence of infections causing only sub-clinical disease makes their diagnosis very difficult. The presence of bacteria in milk taken *via* the teat canal with reasonable aseptic precautions is not sufficient evidence of an intramammary infection since they may be completely derived from the teat canal. If they are associated with an elevated cell count in the milk the likelihood of there being an intramammary infection increases. However, the elevation of cell count may be physiological rather than pathological and with the existing knowledge of the pathogenicity of all intramammary bacteria, particularly the low grade pathogens, the division between the two states must always be arbitrary. The only way that the pathogenicity of bacteria in the mammary parenchyma can be determined is by measuring the pathological effects caused by bacteria known to be resident there. This demands that the infection be confirmed to be of intramammary origin, after which the pathological effects may be related to it. Because of the presence of teat canal infections collection of milk through it is precluded. The insertion of a cannula through the canal and collection of milk after the first flush is of some use (Newbould and Neave 1965) although the passage of an instrument through infected tissue to a possibly uninfected gland has its own dangers. Murphy and Stewart (1954) described a technique for collecting milk by syringe directly from the gland, the needle being inserted through the teat wall into the teat sinus. Bendixen (1932) and Hopkirk (1934) had used a similar technique. While it is difficult to sterilise skin the risks of introducing infection into the gland using this technique with reasonable care are much less than the cannula method.

Undoubtedly this method is the best available to demonstrate definite intramammary infections. It is therefore particularly useful in research investigating the pathogenesis of mastitis.

Part III

The taxonomy of the Micrococcaceae

The Taxonomy of the Micrococcaceae

The classification of staphylococci and micrococci has not been a rewarding subject in as much that a comprehensive scheme has not been developed. An eminent bacteriologist, S.T. Cowan (1962) gave as a major title to a review on the subject "An introduction to chaos". The present author has no real wish to add to the chaos and this section will confine itself to the descriptions given to bacteria of the genera in the literature on mastitis, to state why the descriptions are inadequate and to justify the use of a recently described system (Baird-Parker 1963) as a method to recognise the bacteria for the purposes of the work reported in this thesis.

The recognition of these bacteria became of interest as a consequence of some work prior to that reported here (Forbes and Hebert 1968) which showed that coagulase negative Micrococcaceae, which had been considered non-pathogenic in regard to mastitis were not in fact so when accurate measurements were made of pathogenic effects. There is other literature to support this (McEwan and Cooper 1947, Galli, Gola and Ruffo 1967). When a bacterium is considered to be a pathogen great effort is made to find out which characters account for its pathogenicity. In the work reported (Forbes ~~et al.~~ and Hebert 1968) however some infected quarters showed no evidence of inflammation whereas others did. This made it of interest to be able to recognise the types to determine if the difference in pathogenicity was due to host or parasite variations. Only after that would it be worthwhile attempting to find out what

biochemical system rendered the types pathogenic.

It has been usual in both medical (Cowan 1962) and veterinary literature to designate pathogenic cocci as staphylococci and non-pathogens as micrococci. Coagulase positive staphylococci being the most pathogenic are sometimes referred to as staphylococci and all others micrococci. This takes no account of other differences and is completely unrelated to the taxonomic significance of the two names. In literature on mastitis it is also common for haemolytic strains to be considered pathogens and non-haemolytic strains to be non-pathogens. Some authors describe weakly-haemolytic strains, whose significance can only be guessed. The use of this term is unfortunate for it is probably used to describe a type of haemolysin rather than the production of small quantities. The presence of haemolysed zones around colonies on blood plates can be caused by a number of different haemolysins (Elek and Levy 1950, Fraser 1964) or by a lipase (Orcutt and Howe, 1922). The haemolysins of staphylococci are designated by different Greek letters. Alpha haemolysin acts on erythrocytes of all species although some are more susceptible than others (Bernheimer, 1965). Beta haemolysin acts on erythrocytes of ungulates (Sheep, cow, goat) (Smith and Price, 1938a) and also on human cells; gamma haemolysin on rabbit cells (Smith and Price 1938b) and delta haemolysin on sheep, rabbit, human and horse cells (Marks and Vaughan 1950). Alpha and beta haemolytic cultures are usually also coagulase positive although Kocur, Precechtal and Martinec (1966) found cultures which were not.

The association of coagulase and alpha or beta haemolysins has led to the common use of describing "haemolytic staphylococci" presuming them to be S. aureus. This probably leads to some coagulase positive cultures(which is the most important character of the species) not being recognised (Schalm and Lasmanis, 1957, Ruffo 1966). It is impossible really to assign any significance to the terms haemolytic, non-haemolytic or weakly haemolytic without a fuller description. Weak haemolysis is a term used to describe a narrow zone of haemolysis around a colony grown on blood agar, usually made of sheep or calf blood.

However, the size of the zone is dependent upon the type of haemolysin causing it, the amount produced, the species whose erythrocytes were used in the medium and their susceptibility to the particular haemolysin. If whole blood was used it would also depend on the possible presence of anti-haemolytic antibody in the plasma. Primary culture on sheep or calf blood agar (which are the bloods most commonly used) will not distinguish between alpha and delta haemolysisn although the zone produced by the former is usually, but not always wider. "Weak haemolysis" probably describes delta haemolysis of coagulase negative strains and does not convey any knowledge of pathogenicity although this may eroneously be implied.

Other characters have been investigated as means of differentiating strains. Pigmentation has been studied extensively (Sandvik and Brown 1965, Brown et al 1967) and found to be of little use. This was also the view of Cowan (1962) with the exception of strains producing pink colonies.

Recent trends in the taxonomy of Micrococceaceae are based on fundamental biochemical differences between cultures. Evans, Bradford and Niven (1955) suggested a classification based on earlier work by Evans (1947, 1948) in which Gram-positive and catalase-positive cocci able to utilise glucose anaerobically were placed in the genus Staphylococcus and those unable to do so in the genus Micrococcus. Baird-Parker (1963, 1965a) used the ~~character~~ ability to ferment glucose as fundamental in separating the genera and by examining the nutritional, biochemical and physiological characters of 1250 strains found groups of characters which enabled him to further divide the organisms into subgroups. Originally he used 54 characters and by analysis found that using nine main characters (1965b) a satisfactory scheme for classifying staphylococci and micrococci was obtained. This scheme enabled the Staphylococcus to be subsivided into six subgroups, the first containing coagulase positive cultures, i.e. S. aureus. Micrococcus was subdivided into eight subgroups. Thus it allowed the coagulase-negative Micrococcaceae to be divided into thirteen subgroups based on a short series of biochemical characters. This made it a feasible project to examine sequential isolates from a dairy herd to study the epidemiology of the organisms and is the method used in the work reported in this thesis.

Part IV

General Materials and Methods

The Herd of Cows

The herd on which all the work was done reported in this thesis was composed of monozygous twins. These animals were purchased as calves of a few months of age and reared in isolation from other cows until they were introduced into the dairy herd about a month before the time of their first parturition.

Breeding The cows were served by a bull which was kept exclusively for use on the herd. Service was timed to ensure that the whole herd calved between mid December and March if possible. Some animals with a history of difficult breeding failed to conceive at first service and calved later than this. It was usual to cull both members of a twin pair if for any reason one of them had to be eliminated. However, 1912 and 1933 were allowed to remain in the herd because they were carriers of a strain of S. aureus, the epidemiology of which was being investigated.

Management Throughout two lactations during this investigation the cows were kept at pasture. They were brought into a byre twice each day to be milked. The byre is rectangular with a row of cows on each side tied with their heads facing laterally. In front of each row there is a feeding passage and between them dung channels separated by a central passage 8 feet wide. The entrance and exit is at one end of the building, with the dairy, washing and feeding rooms at the other end.

The positions of the animals during the two lactations are shown in figures three and four. Each animal was tied directly opposite and behind its twin.

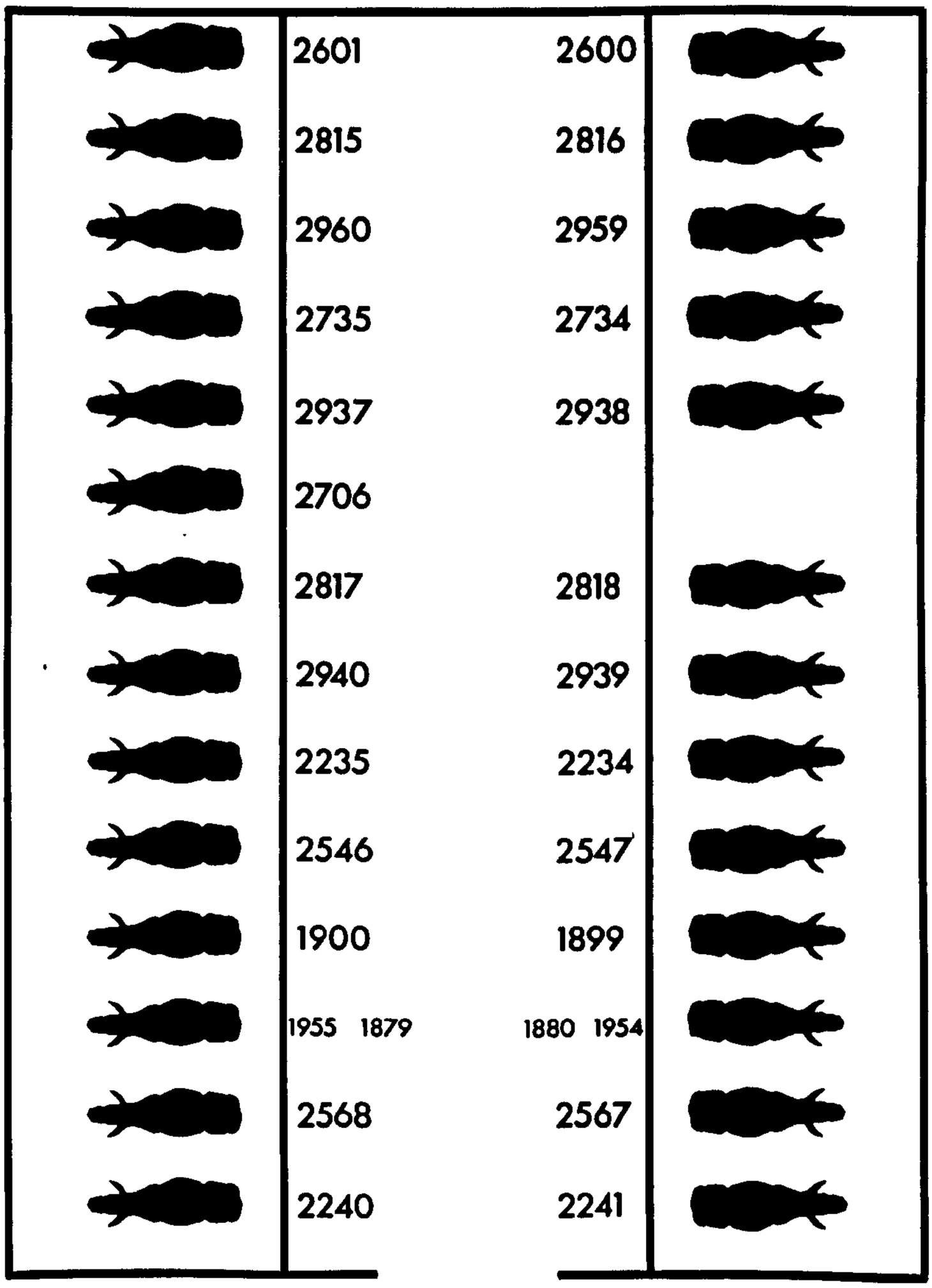


Fig. 3

The standing positions in the byre of cows used in the work reported in Part VI.

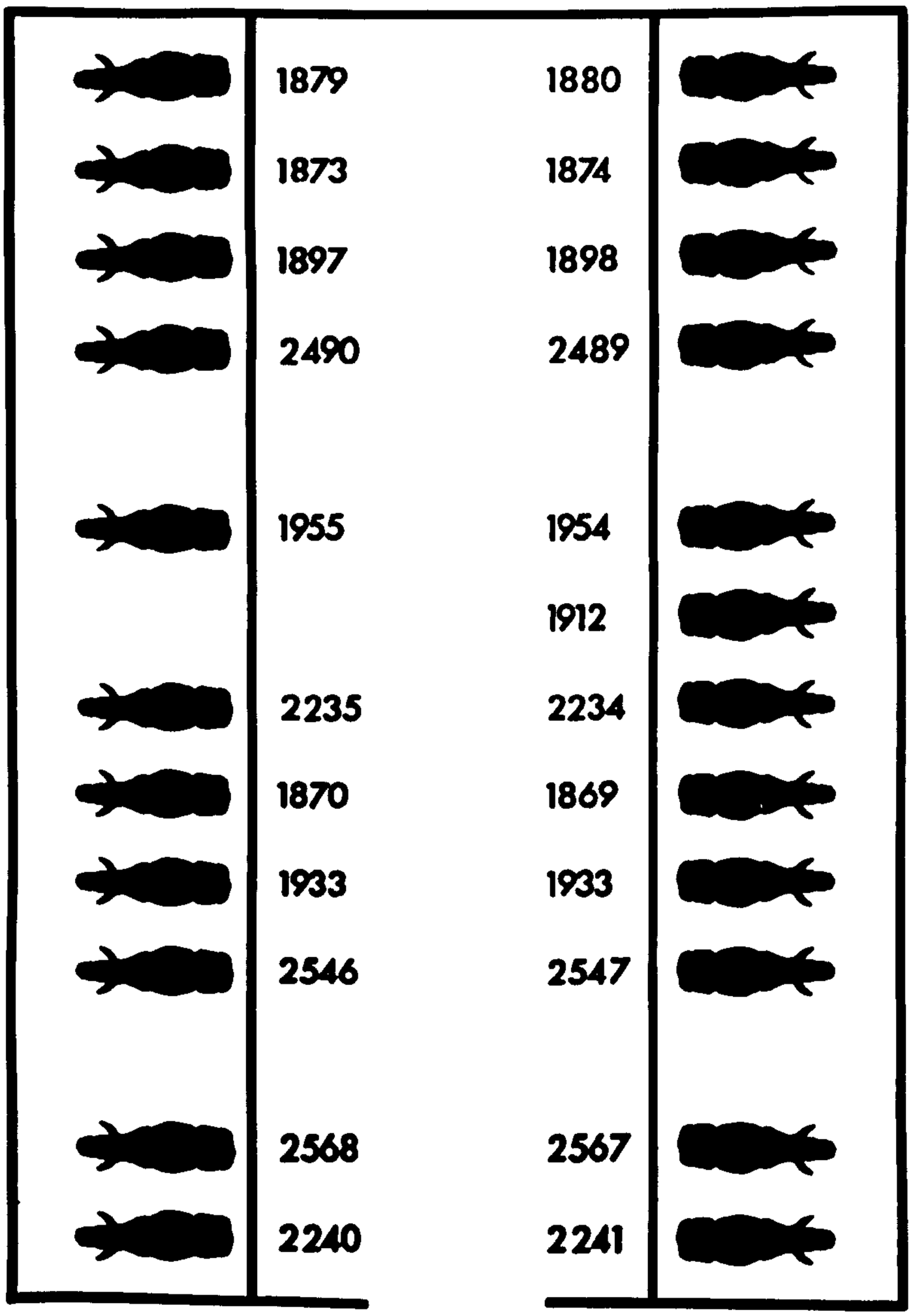


Fig. 4

The standing positions in the byre of cows used in the work reported in Part VIII.

Figure 3 shows the herd used for the work reported in section VI on the epidemiology of teat canal infections. The eight cows at the top of each side of the byre were heifers calving for the first time. The twin of 2706 had to be removed as she was barren. The remaining cows had all had at least one calf previously. Cows numbers 1880 and 1879 had to be removed at the ends of weeks 24 and 25 respectively to make room for numbers 1955 and 1954 which were then newly calved.

Figure 4 shows the herd used for the work reported in section VIII which reports a study in the pathogenesis of mastitis. Eight pairs of monozygous twins and two other cows were used in this work. Cows numbers 1954 and 1955 were dry throughout the period and the results obtained from 1932 and 1933 were not used because of difficulty in collecting milk from them by teat wall puncture. Cow number 2241 calved too late to be included. All the other cows except number 2546 calved within six weeks of each other.

Feeding The cows were at pasture throughout the period. Hay and kale were fed in the fields to supplement their grass diet when necessary depending on the season. Dairy concentrates were fed in the byre in amounts depending on the individuals milk yields. Hay was also available in the byre when the season demanded it.

Milking technique The cows were milked by two operators, each of whom milked one side only. Two Fullwood bucket units were used at each side. The machine maintained a stable vacuum of 13 inches of mercury and the milking units were used with a pulsation rate of 60 per minute and a pulsation ratio of 1:1. After milking was completed all milking utensils were disassembled, thoroughly cleaned and sterilised in a steam chest. The sterile units were reassembled immediately before the next milking.

General precautions No direct contact between the herd and other animals was allowed. No personnel were allowed into the byre except those directly concerned with the herd. The cowmen wore overalls and all other personnel wore waterproof protective clothing which was kept specifically for use in the byre.

Dairy Hygiene Throughout the period disinfectants were not used in the milking technique. The udders were washed in clean water with a separate paper towel before milking. After milking each cow, cold water was flushed through the milking machine cluster via the long milk tube for 15 seconds, (Davidson and Slavin, 1958). The tubes were then reattached and the unit moved to the next cow to be milked.

The cows coats were kept free of grass, dirt and faeces by grooming and washing with water from a hose when necessary.

Collection of samples Milk samples and teat canal swabs were collected immediately before afternoon milking once each week unless otherwise stated. When collecting samples attendants wore protective clothing and rubber gloves. The gloved hands were washed with soap containing hexachlorophane and rinsed in water before each cow was examined. When the sampling procedure was completed the swab inoculated broths and milk samples (see later for detailed descriptions) were immediately taken to the laboratory and the bacteriological examinations started. Media were inoculated with the samples within two hours of collection. Milk samples were then refrigerated overnight when their cell content was to be determined the following day.

Primary isolation of bacteria

0.1 ml of swab inoculated broth or milk were sown onto a plate containing 5 per cent sheep blood agar. The sheep blood was known to be free of streptococcal or staphylococcal antibody. The medium contained aesculin (0.0001 per cent) to facilitate the recognition of aesculin splitting streptococci. It also contained fungistats - i.e. Fungazone (0.01 per cent)* and Actidione$^{\phi}$ (0.0001 per cent). (Baird-Parker 1963)

All plates were incubated at 30°C for 48 hours, and refrigerated overnight before being read. Further identification of the bacteria isolated will be described in detail in the relevant section.

---

* E.R. Squibb, New York.

$^{\phi}$ Upjohn, Kalamazoo.

Cell content of milk

The numbers of cells present in milk samples was determined using and electronic counting method evolved by Cullen (1967b)

Syringe collection of milk by teat wall puncture

The technique described by Murphy and Stuart (1954c)was used to collect milk from the teat simus. The method was adapted slightly. Instead of rubber a nylon fingergrip was used. This was made by cutting a ring from a nylon tubing of a diameter suitable to accommodate the index finger. A hole was punched in the ring through which the ~~barrel~~ plunger of the syringe was pushed up to the glass fingergrip. The ~~barrel~~ plunger was then reinserted into the syringe. Syringes were packed in aluminium cases with a capacity for 100 syringes. The syringes were held in a rack within the case in such a way that the points of the needles were suspended and unable to contact any solid surface. Luer lock fitting syringes were used to avoid needles becoming detached during autoclaving. Fifty syringes were held in each case filling one half of the rack. After a syringe was used it was immediately rinsed and transferred to the other end of the case. This avoided the risk of using a contaminated syringe.

Collection of milk samples using this method is shown in figures 5 and 6. The cows were restrained by holding the heads and elevating the tails. Unlike the original method described, the author preferred to stand with his head forward and leaning against the hind leg of the cow on the same side as the teat from which the samples were being

collected. When samples were collected from a nervous or fractious animal the area of the teat to be perforated was occasionally anaesthetised using local anaesthetic* administered by a Dermo-Jet/ syringe. However, when competence was gained this was seldom done as the collection of the sample was no more painful or traumatic than the administration of the anaesthetic.

---

* Xylotox (Willows Francis)

/ F.H. Wright, Dental Company Ltd. Dundee.

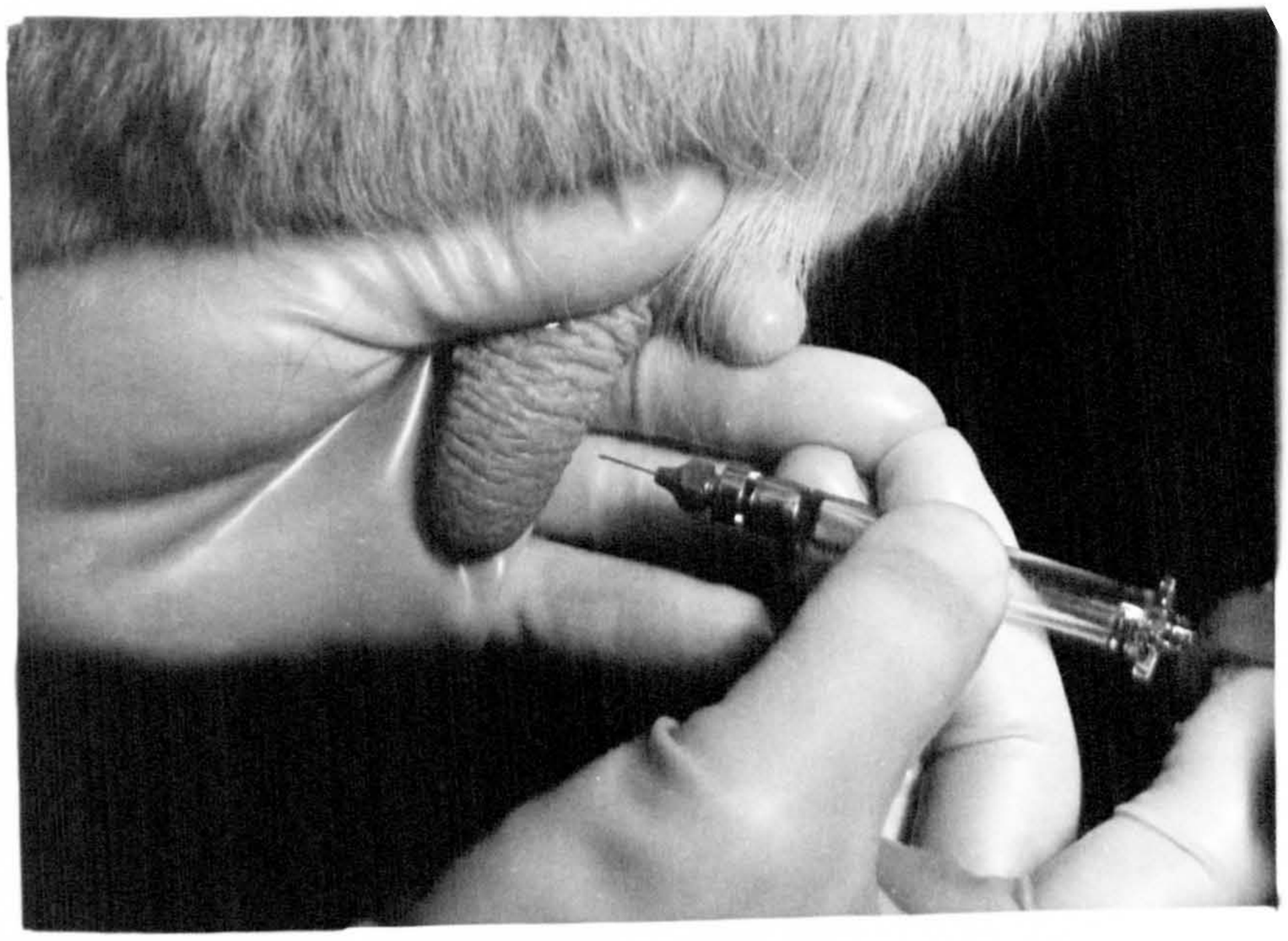


Fig. 5

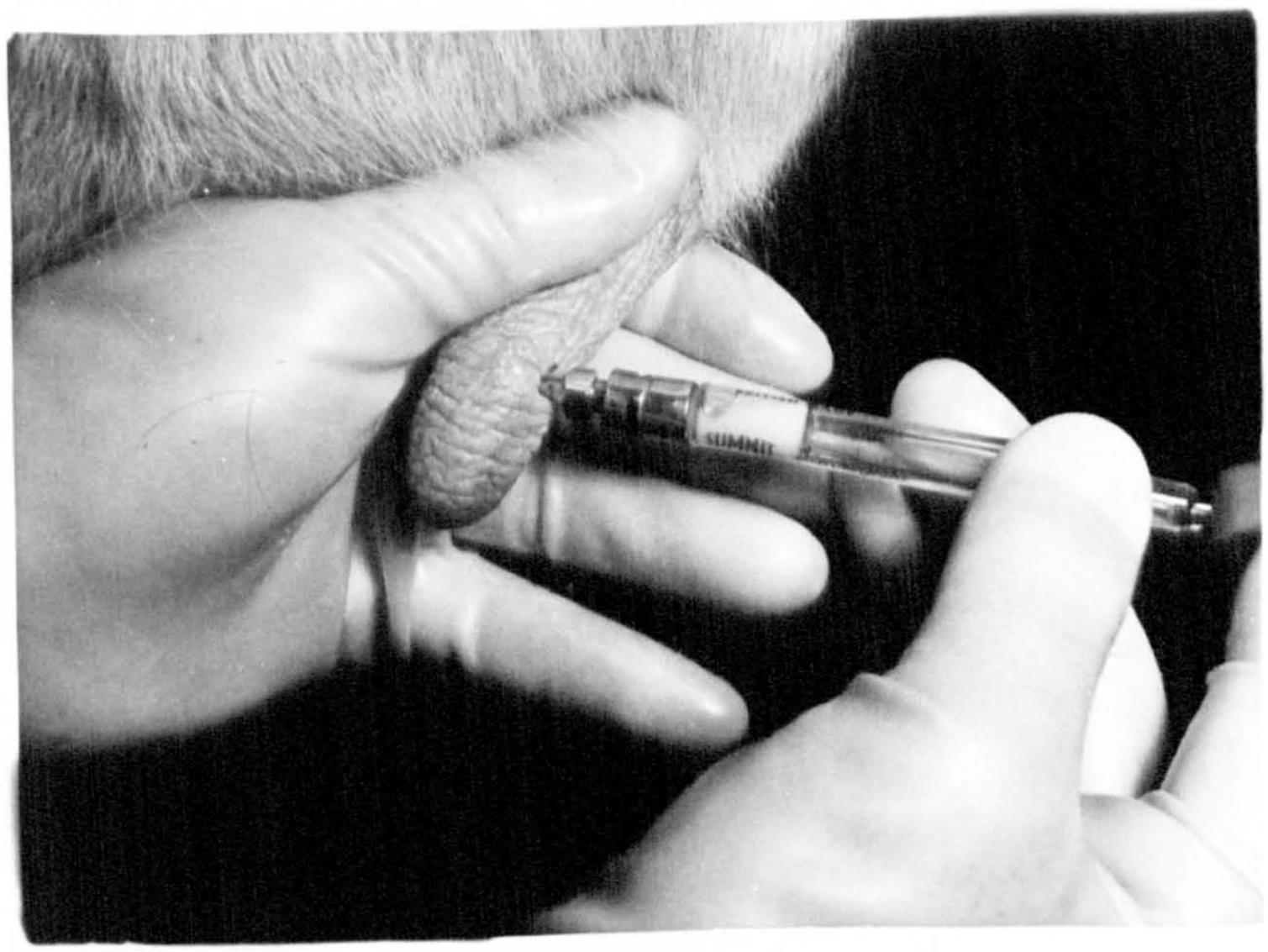


Fig. 6
Collection of milk by syringe through
the teat wall.

Part V

The Taxonomy of some staphylococci and micrococci isolated from bovine teat canals and foremilks

SUMMARY

The taxonomy of 2404 cultures of Gram positive, catalase positive, nonmotile cocci was determined using Baird-Parker's (1963) classification. The bacteria were isolated from bovine teat canals and milk samples during 2 lactations of an experimental herd of cattle and their taxonomic positions were assessed on the basis of 8 reactions. Most were clearly assigned to typical subgroups, although some arabinose positive staphylococci and VP positive and phosphatase positive micrococci were atypical. Cultures representative of these and typical subgroups were examined in more detail using 25 reactions. Most cultures were again classified as being typical but it was shown that the atypical cultures were genuinely different from bacteria examined by Baird-Parker. A marked difference was found in the results of the phosphatase reaction on Baird-Parker's phosphatase medium and on that prepared with a different base used in this work. The atypical micrococci gave a negative result when tested on Baird-Parker's medium but their carbohydrate reactions still differentiated them from other subgroups.

Carbohydrate oxidation tests varied in intensity depending on whether the medium was prepared as slopes or plates. The slopes gave a more intesne reaction which persisted once a positive reaction occurred. The methods used provide additional means for epidemiological studies of Micrococcaceae in dairy herds.

INTRODUCTION

During a study of bovine mastitis (Forbes and Hebert 1968) intramammary infections were confirmed by collecting milk via the teat wall. This established that Gram-positive and catalase-positive non-motile cocci frequently infected the mammary gland and that many of the infections were coagulase-negative types. Davidson (1961a) reported mastitis in a pair of twin heifers in the herd of this laboratory which was caused by coagulase-negative staphylococci. Stabenfeldt and Spencer (1966) described lesions from mammary glands which had shed non-haemolytic coagulase-negative staphylococci. However in the author's investigation, some mammary glands infected with coagulase-negative staphylococci were found to have subclinical mastitis as determined by foremilk cell counts, but others had cell counts similar to those from uninfected quarters. Consequently there was a need to differentiate types in order to establish if the difference was due to variable pathogenicity of the strain or variable host response in the cow. Other workers have recognised a variation in pathogenicity of coagulase negative Micrococcaceae and studied methods of differentiating the strains. Sandvik and Brown (1965) studied the pigments of S. epidermidis by spectrophotometric methods and concluded that strains could be differentiated sufficiently for epidemiological studies. Sandvik and Fossum (1965) classified strains by serological differentiation of their proteolytic enzymes. Both these papers emphasise the need for differentiating strains for the purpose of investigating their association with bovine mastitis. Baird-Parker (1963) introduced a classification of the Micrococcaceae which subdivided

coagulase-negative types into subgroups, depending on their physiological characters. In an attempt to identify strains isolated from the herd so that their pathogenicity and epidemiology could be studied the taxonomy of 2404 cultures was determined. This paper reports the results by using a selection of the reactions described by Baird-Parker (1963). All cultures were examined using 8 reactions and 300 of these cultures were further examined using 25 reactions. All cultures were isolated from milk samples and teat canal swabs taken weekly throughout two lactations of an experimental herd of cows.

METHODS

Isolation of bacteria

During two lactations of an experimental herd of cattle comprised of 23 cows in the first lactation and 13 heifers and 16 cows in the second, the taxonomy of Micrococcaceae isolated each week from teat canal swabs and foremilk samples was determined. Before collecting the samples, the teat skin and apex was thoroughly cleaned with sterile cotton wool soaked in methylated spirits. A swab on a tapered applicator stick was then inserted approximately 3 mm into each teat canal and was rinsed in 5 ml of one per cent glucose broth. 10 ml of foremilk was then collected in a sterile container. 0.1 ml of each broth and milk was sown on a 5 per cent sheep blood agar plate incorporating Fungazone (1.0 per cent) and Actidione (0.001 per cent). Plates were incubated at 30°C for 48 hrs.

Cultures were selected for further examination when at least 25 similar colonies were present on either plate from a quarter. Where mixed cultures were found all types satisfying this criterion, i.e. 2 colonies per plate. were examined. The taxonomic position of the cultures was determined by the following tests; the coagulase test, acid production from glucose in anaerobic conditions, acid production from arabinose, lactose, maltose and mannitol in aerobic conditions, the Voges-Proskauer reaction and phosphatase production. The methods and media specified by the Sub-committee on Taxonomy of Staphylococci and Micrococci (1965) were used. Representative cultures of each subgroup were more comprehensively examined with additional tests. The details of all tests follow.

Colonies from the primary plates were subcultured on Tryptone yeast extract agar (Difco) at 37°C.

Motility was tested in a drop of beef infusion broth incubated 16 hrs. at 37°C.

Morphology was examined on a smear prepared from the broth cultures and stained by Gram's stain.

Catalase test Nutrient agar slopes were inoculated, incubated at 37°C for 24 hours.and 1 ml. of 20 volumes H_2O_2 was run over the slope. Immediate evolution of gas indicated catalase activity.

Coagulase The production of free coagulase was determined in 1 ml of a 1 in 5 dilution of fresh rabbit plasma which was incubated at 37°C for 24 hours. (Subcommittee on Taxonomy of Staphylococci and Micrococci (1965)). The production of bound coagulase was determined with the slide test,(Duthie, 1954).

Acid Production from Carbohydrates The glucose medium was dispensed in tubes 16 mm x 120 mm and were steamed before inoculation to expel oxygen from the medium. Anaerobiosis was ensured by incubating in anaerobic jars. The cultures which were examined in detail were also inoculated into another tube of glucose medium which was incubated aerobically. The modified Hugh and Leifson medium as described by Baird-Parker (1963) was used in all other carbohydrate tests. Fourteen carbohydrates were used, namely L-arabinose, cellobiose, dextrin, galactose,α-methyl-D-glucoside, glycerol, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin and xylose. The carbohydrates reactions were tested on slopes in 5 ml vials with screw caps, which were

loosely applied during incubation. Some were also tested on agar plates onto which two cultures were streaked.

Lipolysis Ability to hydrolyse butter fat to release free fatty acids was tested on the medium of Jones and Richards (1952) incubated at 37°C. Plates were read at 24 hours and at 5 days.

The media and reagents used in the tests listed below are those described by Cowan and Steel (1965) unless otherwise stated.

Starch hydrolysis Starch agar plates were inoculated, incubated 5 days and tested by flooding with Lugol's iodine solution.

Phosphatase Phenolpthalein phosphate agar plates prepared with Oxoid No.2 nutrient agar were incubated for 24 hours before exposing to ammonia vapour.

Casein digestion Casein agar plates were incubated for 5 days. Each plate was then flooded with acid mercuric chloride solution to distinguish between true proteolysis and clearing of the milk by other products.

Ammonia production from arginine broth - Broths were incubated for 14 days (Baird-Parker 1963) and tested for ammonia production with Nessler's reagent.

Nitrate reduction Nitrate broth was tested after 14 days incubation. Reduction of nitrate was determined by adding 1 ml. of each of the Griess-Ilosvay reagents to the broth. Reduction beyond nitrite was tested by adding zinc dust.

Voges-Proskauer reaction After 14 days incubation the presence of acetoin was determined (Barritt 1936).

Phage Typing Some cultures were phage typed with the phage set of Davidson (1961b).

Analysis of results Positive and weak positive reactions were punched onto cards which were then sorted into subgroups on the basis of Baird-Parker's classification.

The subgroups were not given the identity of species by Baird-Parker but simply numbered as six staphylococcal subgroups and eight micrococcal subgroups. His paper (1965b) called the first staphylococcal subgroup "S. aureus" and the other subgroups Staphylococcus epidermidis subgroups 1 to 5. In this thesis the coagulase-positive cultures (S. subgroup 1) are called S. aureus and the other subgroups simply by their original numbers (Baird-Parker 1963), e.g. S. subgroup 2, M. subgroup 1. etc.,

RESULTS

During two lactations the taxonomy of 2404 cultures were examined, 2361 of which were coagulase negative when tested for free coagulase with the tube test. 170 of the latter cultures, all isolated during the first lactation had other characters identical to that expected from Staphylococcus aureus. Fifteen of these cultures were positive for bound coagulase with the slide test (Duthie 1954). Occasional isolates from the same teats as the majority of these cultures also produced free coagulase. When phage typed they were weakly lysed by phages 53 and 31B. The fifteen cultures producing bound coagulase were also lysed by these phages. They also produced α haemolysin when tested using the method of Elek and Levy (1950) although haemolysis was only detectable in rabbit blood agar plates. These additional reactions showed these cultures to have several characters typical of S. aureus, even though they are unable to produce free coagulase.

Coagulase negative Micrococcaceae - The characters of the remaining 2191 cultures negative to the tube coagulase test were used to divide the cultures into subgroups and are shown in Table 1.

Staphylococcus subgroups Staphylococcus subgroup 2 includes some cultures which oxidise mannitol. Their ability to utilise mannitol anaerobically was not determined and these cultures may therefore be coagulase negative S. aureus types. Baird-Parker (1965a)suggested that cultures should be classified as S. aureus if they were positive to two of three specified reactions, namely the coagulase test, phosphatase test and anaerobic

Table 1

Characters of subgroups expressed as the percentage of positive reactions

	Staphylococcus subgroups								Micrococcus subgroups							
	1	2	3	4	5	6	Atypical Staphylococci		1	2	3	4	5	6	7	9
Glucose Anaerobic acid production	100	100	100	100	100	100	100	100	0	0	0	0	0	0	0	0
Free coagulase production	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bound coagulase production	100[9]*	NT+	NT	NT	NT	NT	0[18]	0[10]	NT	NT	NT	NT	NT	NT	NT	0[23]
Phosphatase production	100	100	100	0	0	0	0	100	0	0	0	0	0	100	0	100
Acetoin production	100	100	0	100	100	99	100	100	100	100	100	100	0	0	0	100
Aerobic acid production from Arabinose	0	0	0	0	(11)++	0	100	100	0	4	0	100	10(11)	(13)	0	45
Lactose	94	89	93	0	100	67(3)	100	100	0	50	67	100	53(21)	36(12)	0	63
Maltose	99	100	16	100	100	68	100	100	45	100	97(2)	100	36(43)	29(11)	0	91
Mannitol	100	23	8	0	0	18	100	100	0	0	100	100	51(5)	23(19)	0	78
Total number of isolates	170	255	146	2	36	246	85	203	15	24	286	33	176	379	105	200

The figures quoted are the percentage of the cultures examined showing a positive response to the test indicated

*The figures quoted in brackets are the percentage of cultures in each subgroup tested for bound coagulase

+ The figures quoted in parentheses are the percentage of cultures showing a weakly positive response to the test indicated.

utilisation of mannitol. Staphylococcus subgroup 3 includes some cultures which oxidise maltose and mannitol in addition to lactose, characters by which they differ from bacteria of this subgroup examined by Baird-Parder. They were all phosphate positive and Voges-Proskauer negative and fermented glucose when incubated 5 days, characters by which they were included in this subgroup.

There were 246 cultures placed in Staphylococcus subgroup 6, 3 of which were Voges-Proskauer negative. These three were phosphatase negative and produced acid from mannitol aerobically, two characters typical of the group. Columns 6 and 7 of the table include staphylococci which oxidised all the four sugars tested. Some were phosphatase negative and others were phosphatase positive. Their reaction with arabinose differs them from any staphylococci examined by Baird Parker. Micrococcus There were 1218 cultures which had reactions which clearly assigned them to typical subgroups. One hundred and five cultures did not react with any of the test media and are recorded as subgroup 7. One hundred and forty cultures which did not form acid from glucose anaerobically were phosphatase positive and Voges-Proskauer positive. In addition most utilised lactose, maltose or mannitol, some reacting with all three carbohydrates. These 140 cultures are not typical of any Baird-Parker subgroup.

Results from the detailed examination of 300 cultures

Cultures which were representative of typical subgroups and in addition 43 S. aureus cultures were subjected to more detailed

examination using 25 reactions. The results are shown in Table 2.

Staphylococcus

S. aureus Forty-one cultures produced free coagulase. Two other cultures had reactions typical of S. aureus but produced only bound coagulase. Three cultures were negative to the Voges-Proskauer test and two of these were the only cultures to reduce nitrate beyond nitrite.

S. epidermidis Most staphylococcal cultures had reactions which clearly assigned them to typical Baird-Parker subgroups. Cultures of the arabinose positive staphylococci were also tested using these reactions. These results are included in Table 2. The staphylococci were able to oxidise a wide range of carbohydrates by which they were further differentiated from previously reported staphylococci. All but one of these cultures were phosphatase positive. Five of the cultures were retested using the full series of carbohydrates on plates to determine if their reactions differed from those obtained on slopes. During five days incubation the tests were read daily and the reactions were similar on both plates and slopes although on plates a positive reaction occasionally changed back to negative during the period. The main difference in the tests were the arabinose reactions. On slopes a positive reaction was present and persisted after 48 hours.incubation whereas on plates there was no evidence of acid production.

Micrococcus All but eight of the micrococci had reactions which clearly assigned them to subgroups. The eight cultures were those which gave positive reactions to the phosphatase and Voges-Proskauer tests. These

Table 2. Complete characters of subgroups expressed as the percentage of positive reactions

	Staphylococcus subgroups				Atypical Staphylococci		Micrococcus subgroups						
	1	2	5	6			1	2	3	5	6	7	9
Glucose Aerobic acid production	100	100	100	93(2)*	100	100	0	27(73)	51(49)	12(88)	30(70)	0	0
Glucose Anaerobic acid production	100	100	100	100	100	100	0	0	0	0	0	0	100
Free Coagulase production	95	0	0	0	0	0	0	0	0	0	0	0	0
Phosphatase production	100	100	0	0	0	100	0	0	0	0	100	0	(13)
Acetoin production	93	91	100	100	100	100	50	100	97	0	0	0	0
Aerobic acid production from													
Arabinose	0	0	0	0	100	70(25)	0	0	0	8	40	0	0
Cellobiose	0	0	0	0	100	100	0	0	0	0	0	0	0
Dextrin	0	0	0	0	100	100	0	0	0	0	0	0	100
Galactose	86	82	75	54	100	100	0	73	43	19	70	0	0
Alpha-methyl-D-Glucoside	5	0	0	1	100	(10)	0	0	0	0	0	0	0
Glycerol	100	100	100	100	100	100	0	100	100	88	90	0	38(62)
Inositol	0	0	0	0	100	100	0	0	0	0	0	0	62(38)
Lactose	98	100	100	96	100	100	0	100	89	81	90	0	0
Maltose	95	100	100	98	100	100	100	91	82	85	45	0	0
Mannitol	100	0	0	100	100	95(5)	0	0	96	69	65	0	25
Raffinose	0	0	0	0	100	80(15)	0	0	0	0	0	0	13
Rhamnose	0	0	0	0	100	85(15)	0	0	0	0	0	0	100
Salicin	0	0	0	0	100	100	0	0	0	0	0	0	100
Xylose	0	0	0	0	100	85(15)	0	0	0	15	35	0	100
Clearing of Casein	16	27	69	3	0	5	50	82	11	19	50	100	100
Nitrate													
No reduction	0	18	3	28	0	0	0	0	28	31	0	33	100
Reduced to nitrite	95	82	97	63	100	100	50	100	64	50	90	33	13
Reduced beyond nitrite	5	0	0	8	0	0	50	0	7	19	10	33	0
Ammonia from Arginine broth	100	100	100	100	100	100	0	100	100	88	85	0	100
Butter hydrolysis	60	100	100	86	0	100	0	100	64	35	90	0	100
Diastase production	0	0	0	0	0	0	0	0	0	0	0	0	0
Total number of isolates	43	11	32	95	1	20	2	11	28	26	20	3	8

*% of isolates weakly positive

cultures were also different from any other micrococci tested in that they produced acid from dextrin, salicin and rhamnose. However, their principal difference was that they were positive to the phosphatase and Voges-Proskauer tests. Some of these cultures were examined by Dr. A.C. Baird-Parker (Unilever Research Laboratory) who found them to be phosphatase negative on his medium. The cultures were then retested on phosphatase plates prepared by Baird-Parker's methods and on these their reactions were weak or negative. Subsequent tests showed that the difference was in the basal medium used for the plates, as otherwise the preparation of the two media was identical.

DISCUSSION

Most cultures classified by examining 25 characters fell into groups proposed by Baird-Parker (1963 and 1965). S. aureus cultures differed from his results in the nitrate test as only two reduced nitrate beyond nitrite. Both these cultures were Voges-Proskauer negative which is also atypical of the species. Two cultures with reactions otherwise typical of S. aureus did not produce free coagulase. When cultures were examined using eight characters, 170 cultures had reactions typical of S. aureus with the exception of free coagulase production. Some of these cultures were tested using the method of Elek (1950) and produced α-haemolysin which was detectable on rabbit blood agar. This feature allied to the susceptibility of these cultures to phages 31B and 53 and the production of bound coagulase classifies them as S. aureus. As the 170 cultures were mostly recurrent isolates from the same sources all are considered to be S. aureus.

Staphylococcus subgroup 2 includes some cultures which utilised mannitol aerobically. Their reaction with mannitol anaerobically was not determined and these cultures may therefore be coagulase negative S. aureus types since Baird-Parker (1965a)suggested that cultures which had two positive reactions from the coagulase test, phosphatase test and fermentation of mannitol should be classified as S. aureus.

Only 23 of the 52 cultures classified as Staphylococcus subgroup 3 were typical. The remainder formed acid from either maltose or mannitol. None of these atypical cultures were tested using the results of 24

reactions. The cultures forming acid from maltose or mannitol in addition to lactose differed them from Micrococcus subgroup 6 only in their anaerobic utilisation of glucose. Five replicate glucose tubes of 3 cultures from each of these two groups were inoculated. Those inoculated with Micrococcus subgroup 6 cultures were negative when read at both 5 and 10 days. The Staphylococcus subgroup 3 reactions were not uniform. Some tubes had acid throughout the length of the tube at 5 days while others had acid in the upper half only. At 10 days all had acid throughout the length of the tube. Baird-Parker (1963) advocated reading at 10 days but the Subcommittee on Taxonomy of Staphylococci and Micrococci (1965) specified 5 days incubation. In this experiment the results were more repeatable at 10 days. Incubating in anaerobic jars also indicates some convergence between staphylococci and micrococci. Some micrococci caused colour changes from the original purple to less intense shades which indicates some degree of acid formation. In this work all cultures classified as staphylococci produced acid throughout the length of the tube after 5 days incubation. Kocur and Mortensen (1967) tested several media and concluded that the division of staphylococci and micrococci on the basis of anaerobic production of acid from glucose is not completely satisfactory although the methods recommended by the Subcommittee on Taxonomy (1965) if adhered to, are probably the best possible. Gibson (1967) agreed with this although strains showed a varying capacity to grow anaerobically at the expense of glucose fermentation and therefore the method lacks precision

as a criterion for differentiating between the two genera. Brown, Sandvik, Scherer and Rose (1967) reported two serological groups of Staphylococcus epidermidis (groups G and NRO)which had similar reactions to those maltose and mannitol positive Staphylococcus subgroup 3 reported here .

During this investigation cultures were examined whose characters were not typical of any of the subgroups described by Baird-Parker (1963, 1965a.) They were arabinose-positive staphylococci and phosphatase and Voges Proskauer positive micrococci. The staphylococci had two strains determined by the eight reactions, varying only in their phosphatase reaction. Both types were examined more comprehensively (Table 1) and apart from the phosphatase test had the same reactions, being particularly active in the range of carbohydrates they were able to utilise. As these types were first isolated from the herd at about the same time it is probable that they are related.

The phosphatase reaction was determined using nutrient agar (Oxoid) as the basal medium. This base was compared with that used by Baird-Parker 1963 and found to be more sensitive. Brown, Sandvik, Scherer and Rose et al (1967) also recorded a greater sensitivity in a broth phosphatase test than Baird-Parker's test but concluded that Baird-Parker's method may be better because weak phosphatase producing cultures then appear negative, permitting a definite positive or negative classification. The sensitivity of the phosphatase test used in this work differentiated a group of micrococci which were phosphatase and Voges-Proskauer positive. On Baird-Parker's

phosphatase medium they gave negative results and on the basis of eight reactions could then be classified in Micrococcus subgroups one to four. However, using the full series of carbohydrate tests their reactions are more typical of Micrococcus subgroup 5 but are excluded from this group by their reaction to the Voges-Proskauer test.

A difference in sensitivity was also evident in the carbohydrate utilisation tests when used on slopes instead of plates. The reaction on the slopes was more intense and moreover was permanent once a positive reaction occurred. The arabinose positive staphylococcus type was recognised on slopes although it would have been missed using plates as the reaction was then negative. However, the thirteen other carbohydrates tested on both plates and slopes, showed the cultures to be able to utilise a large range of carbohydrates, unlike other staphylococci. (Table 2).

Throughout this work cultures on primary isolation were chosen for identification by their colonial characters and pigmentation. In mixed cultures each type present was examined. It was found that colonies with different pigments often had identical biochemical reactions and therefore the character was not recorded. Unless complicated methods such as those used by Sandvik and Brown (1965) are used, pigmentation is of little use in differentiating types, but Brown et al (1967) found the method was valuable in showing persistent infections of the same type of organism in particular quarters of an udder. The routine methods used in this work give results sufficiently accurate to identify strains.

Terplan and Gedek (1965) and Wilssens, Casteele and Demeter (1966) reported the study of staphylococci and micrococci isolated from milk using Baird-Parker's classification. The former workers isolated only one Micrococcus from the 102 cultures examined, whereas the proportion of micrococci and staphylococci reported here were similar. The incidence of infection would suggest that the pathogenicity of micrococci should not be ignored, particularly as Mitchell (1965) found Micrococcus subgroup 3 to be a primary pathogen in human urinary tracts.

Both S. aureus and coagulase negative Micrococcaceae share similar environments on cows udders and teat skin (Davidson 1961a; Cullen and Hebert 1967) from where penetration into the mammary gland may occur ~~et al~~ and Hebert (Forbes/1968). They must also be subject to the same vectorial transmission within a herd. This examination of Micrococcaceae was not intended primarily as a taxonomic study but as a means of differentiating the bacteria sufficiently to allow their epidemiology to be followed. This was practicable and the epidemiology of strains within the herd will be reported in Part VI.

Part VI

The epidemiology of teat canal infections in a dairy herd

Summary

The teat canals of an experimental herd of cows were swabbed weekly throughout a 40 week period. Micrococcaceae isolated from the swabs were typed using the classification of Baird-Parker (1963). S. aureus cultures were typed using a provisional international phage set for typing bovine staphylococci (Davidson 1967). The epidemiology of the bacterial subgroups are presented on maps which show the distribution of the bacteria in the herd in relation to the standing positions of the cows in the byre throughout the period. These showed the directions of spread of infections which were unrelated to the milking procedure. There was no significant difference in the frequency that bacteria spread along the direction of the milking line to the frequency they spread against it. Infections also passed across the byre, probably transferred by contact between cows in the central passage when released after milking. Infections tended to spread progressively to the animals in closest proximity to that from which the infection was first isolated. The distribution of cultures with similar characters provided evidence of taxonomical similarities between some of Baird-Parker's subgroups.

Introduction

In mastitis control programmes in which the prevention of transfer of bacteria from one cow to another is an important part various disinfectant procedures are used. The vectors which are incriminated are contaminated milking machines, udder washing cloths and milkers hands. Davidson and Slavin (1958) devised a technique which removed up to 99 per cent of bacteria from the teat cups of milking machines between cows. Other procedures such as washing of the udder or dipping the teats in disinfectant solutions are common practice. Davidson (1961_a) provided evidence that social contact between cows was a factor in the transfer of bacteria. It has been argued elsewhere in this thesis that of all infections skin bacteria are most commonly found within the mammary gland. Consequently a detailed examination of bacteria resident in the teat canal was made. It was evident that the bacterial populations were not static and fluctuated in both incidence and and type during the course of the lactation of a dairy herd. Their distribution of each type has been examined and interesting patterns of spread within the herd observed. This section reports these findings.

Materials and Methods

The bacteriology of teat canals of a herd of dairy cows was examined during 40 weeks, during which time all cows were in milk although some had not calved at the beginning of the period.

Teat canal swabs The teat canals were swabbed at weekly intervals in all animals, whether or not they had calved. The collection, culturing and examination of the bacteria is described in Part V. Cultures were tested only when 25 or more similar colonies were present on the plate used for primary isolation. At least 1250 colony forming units of each culture had therefore been obtained from the original teat canal swab.

Coagulase positive cultures (S. aurcus) were typed using a provisional international phage typing set for bovine staphylococci proposed by Davidson (1967).

Methods of analysis

The positive characters of each culture were punched onto cards along with the number and quarter of the cow from which it was derived and the number of the week it was isolated. The cards were then sorted into groups according to the biochemical reactions. By this means the subgroup of each culture was determined and the time of isolation recorded.

Presentation of the epidemiological patterns of each bacterial subgroup

The figures in the results section which follows show the distribution of all the bacteria within the herd in respect of the positions the cows stood in the byre at milking time (See figure 3). The figures show the distribution in relation to position and time. The cows on

each side of the byre stood with their heads tied laterally. The four quarters of the cows on each side of the byre are represented each week as shown in the key below the figures. To show the progression of the infections the week numbers of lactation progress laterally to each side of the figures.

When a culture was isolated from a teat canal on a particular week the relevant corner of the graph paper was filled by a closed or an open square. The bacterial subgroup each square represents is shown in the caption under each figure.

Some cows were lactating at the start of the experiment: the time at which the others calved is shown by a vertical black line.

Sampling of the herd was continued weekly for 40 weeks and the figures are adjusted to show the first occasion a bacterium of each subgroup was isolated.

Results

The numbers of each bacterial strain isolated each week varied during the lactation. They are shown graphically in figures 7 (Staphylococci) and 8 (Micrococci). The number of cultures of each type isolated each week are plotted and the time scale related to the time of year.

Figure 7 shows that at the beginning of the lactation S. subgroup 3 predominated and its incidence subsided gradually during the lactation. The atypical staphylococci are designated in the graph S7 (phosphatase negative) and S8 (phosphatase positive). Both types were first isolated within two weeks of each other and by the end of lactation together were the predominant staphylococci. The incidence of the other staphylococcal subgroups fluctuated about a low mean although S. subgroup 6 was not isolated at all during a period in mid-lactation.

In figure 8 it will be seen that M. subgroup 6 and M. subgroup 3 predominated early in lactation, the former prepondering throughout the period. The other subgroups fluctuated about a low mean although the atypical micrococcus, M. subgroup 9 increased in incidence at the end of lactation.

The distribution of S. subgroup 3 and M. subgroup 6 followed similar trends and the significance of this will be discussed later.

Epidemiology of bacteria in the herd

S. aureus (Staphylococcus subgroup 1. Baird-Parker)

There were two strains of S. aureus in the herd. Strain A had the phage pattern 29, 42E, 101, 11077AC1 3A, M8, Seto 1, Seto 6, 6, 75+ and Strain B, AC1, Seto 1.

Fig. 7

Incidence of coagulase negative staphylococcal subgroups

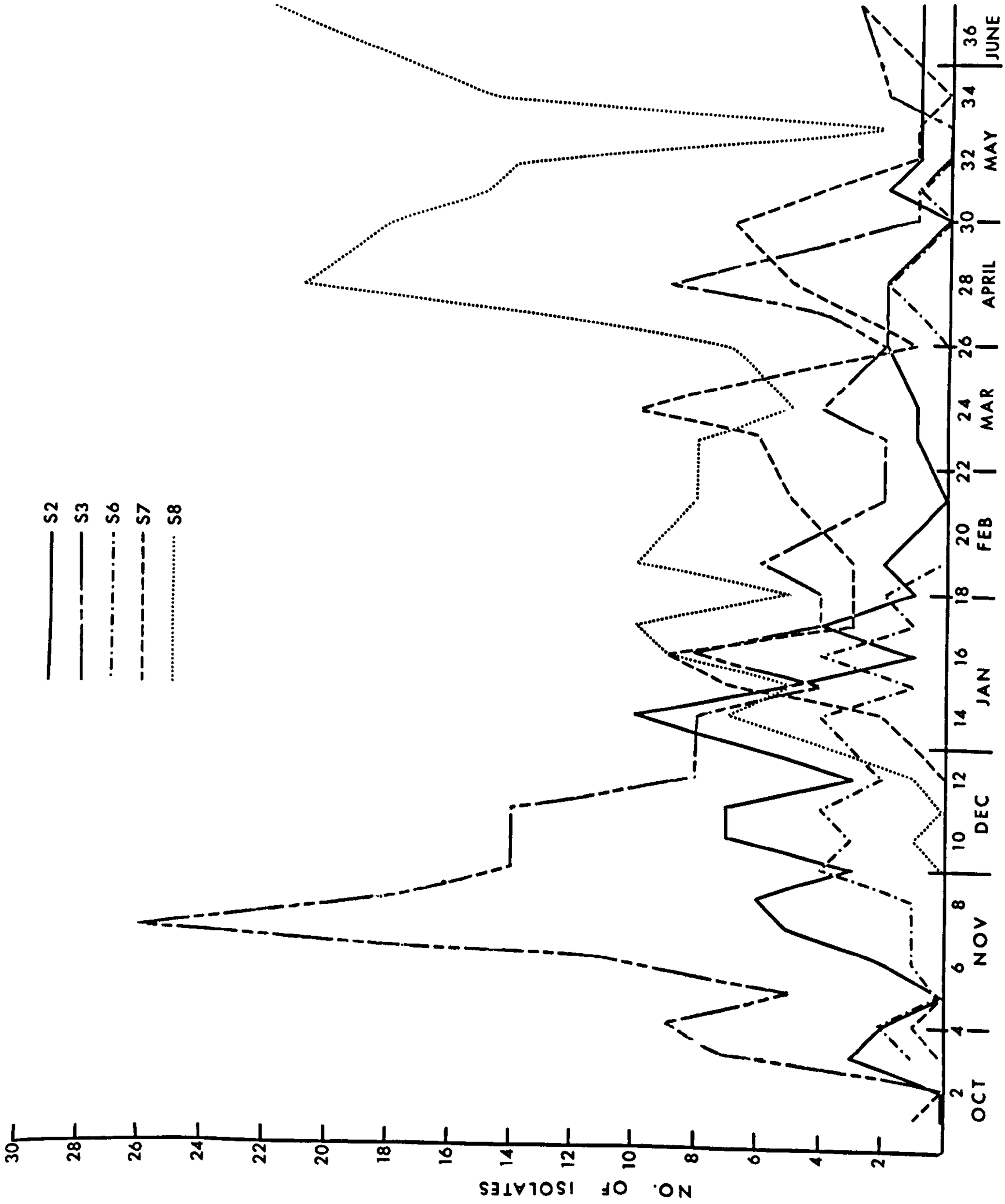
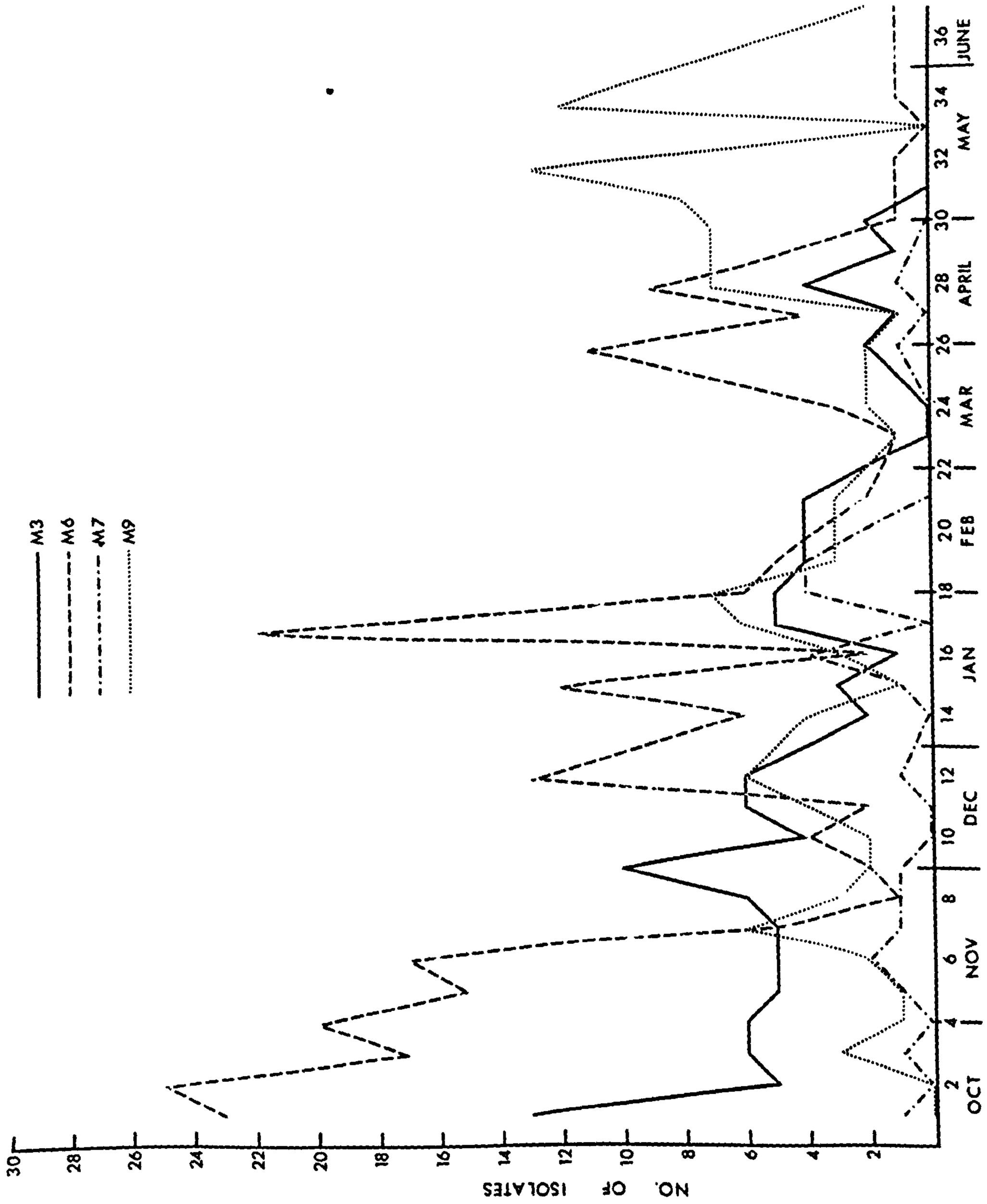


Fig. 8

Incidence of micrococcal subgroups



The distribution of the two strains is shown in figure 9. Neither strain was isolated from a cow before calving but both were isolated from the cows at the lower end of the byre in week 3. Both strains were isolated in later weeks in a manner suggesting gradual spread up the line of cows on each side of the byre. Strain A was first isolated from the heifers in cow number 2706 on the left side of the byre, at week 8, then from 2960 at week 15 and reached 2601 at the top of the byre at week 18. In the same week it was also isolated from her twin on the right side of the byre and only one other teat canal on this side had been previously infected, the right fore teat of 2938.

Strain B was isolated sporadically from 2 cows 2240 and 1899 in the first weeks of lactation. The heifer 2735 became infected in one teat canal in week 8 and two others from week 9. At week 11, 2959, an animal on the opposite side and above 2735 had the bacterium in the left hind teat canal. The strain was periodically isolated from cows below and above these animals in a manner which suggests the bacterium was being disseminated from them.

Staphylococcus subgroup 2

Two types of bacterium were placed in S. subgroup 2. The mannitol positive strain, being maltose negative cannot be considered an aberrant strain of S. aureus. The two types are shown in figures 10 and 11. Their distribution does not suggest they are related as in general they were isolated from different cows. The numbers of each culture isolated were small and were widely distributed in time and location.

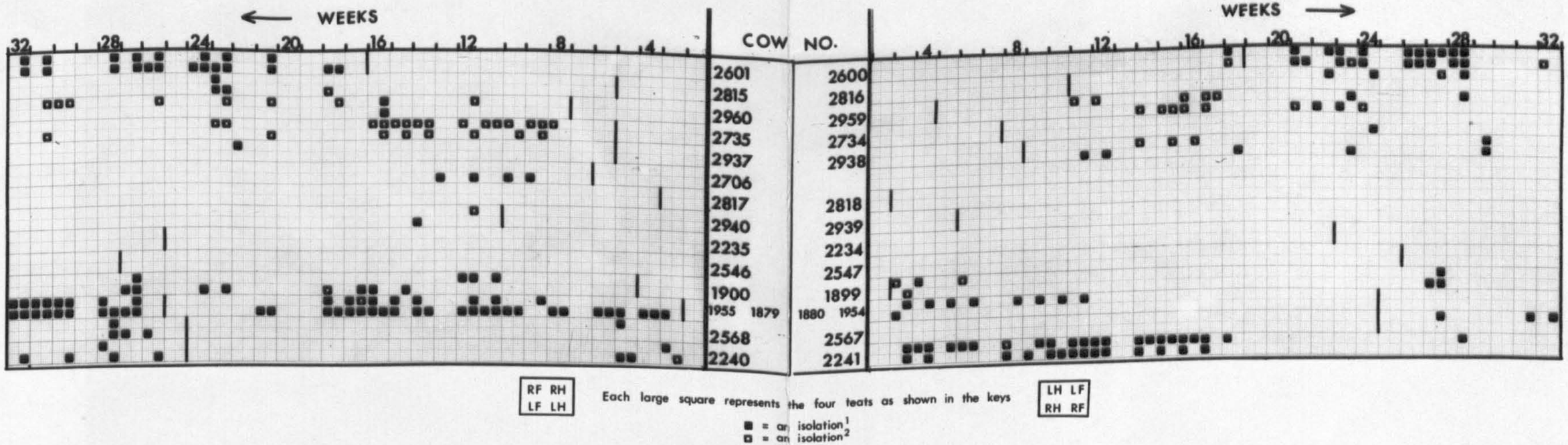


Fig. 9

Distribution of S. aureus

1 = Strain A (Phage type 29/42E/101/110/77ACL/3A/MB/Seto 1/Seto 6/6/75+)

2 = Strain B (Phage type ACL/Seto 1)

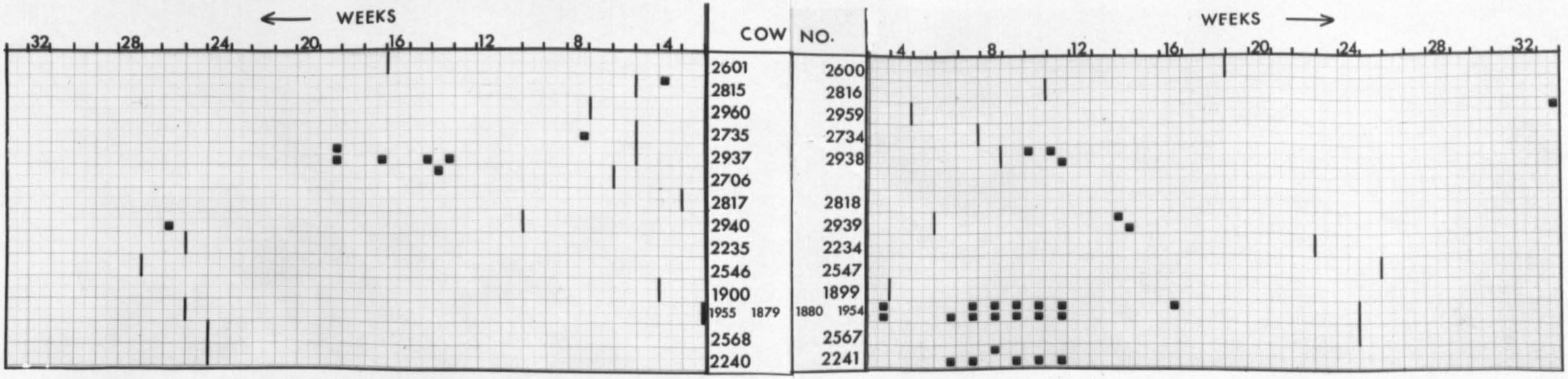


Fig. 10

Distribution of S. subgroup 2 (maltose -ve)

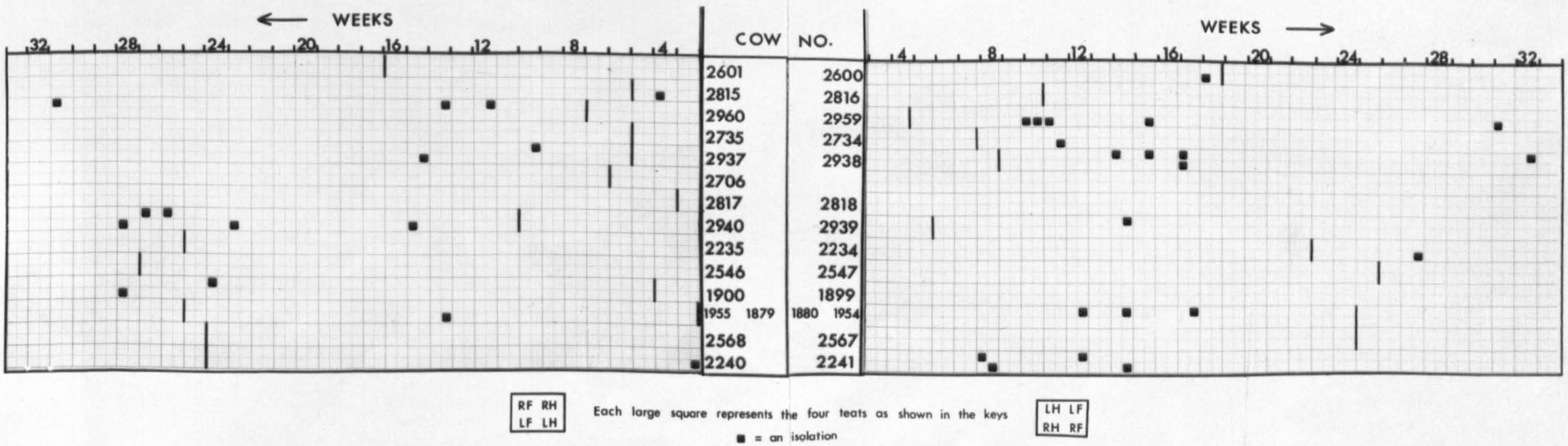


Fig. 11

Distribution of S. subgroup 2 (maltose -ve, mannitol +ve)

Staphylococcus subgroup 3.

The distribution of cultures of S. subgroup 3 which were typical of Baird-Parker's subgroup are shown in figure 12. The first isolation was in week 1 from cow 2601 and after that it was isolated from other cows on the same side. On the right side it was first isolated from 2734 on week 4 after which there were occasional isolations from cows on each side of her.

S. subgroup 3 maltose positive cultures are shown in figure 13. Cultures were first isolated in week 3 on both sides of the byre and were widely separated.

Mannitol positive S. subgroup 3 cultures are shown in figure 14. The first isolation was from 2959 in week 3 and then from her twin on the other side of the byre in the next week. There was some evidence of subsequent spread up and down each side of the byre from these cows.

Staphylococcus subgroup 6

The first isolation of a culture in this group was in week 3 from 2816 and in the next week from 2240 from the opposite corner of the byre (Figure 15). The few isolations were widely separated throughout the lactation, only 2939 having a continuous infection but of short duration.

Staphylococcus subgroups 7 and 8

These cultures are those which are different to any described by Baird-Parker. S. subgroup 7 was isolated in week 11 from 2939 and from

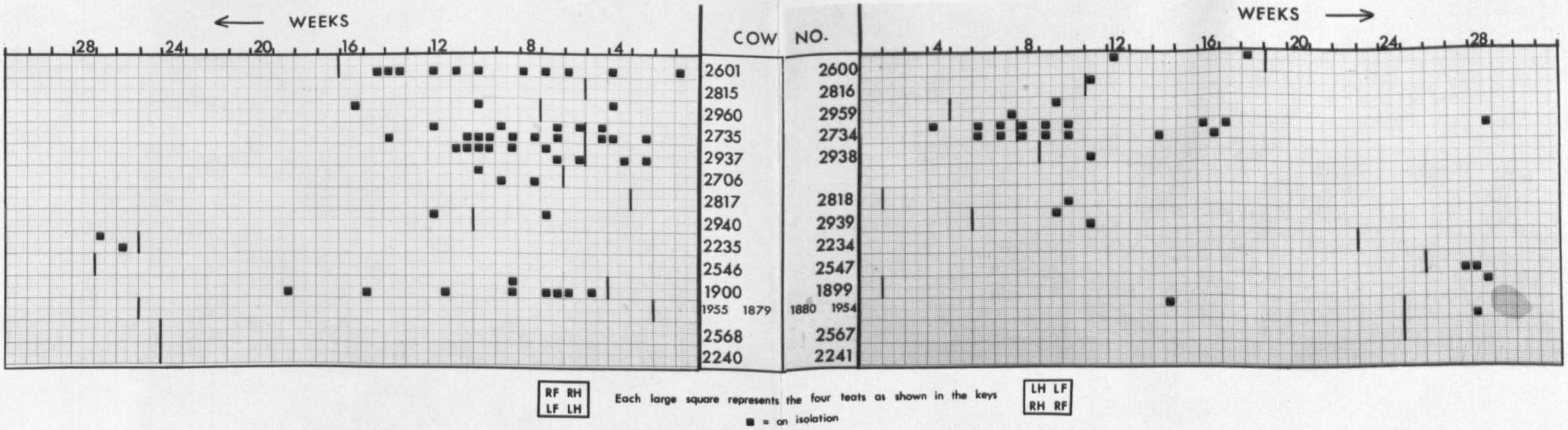


Fig. 12

Distribution of S. subgroup 3.

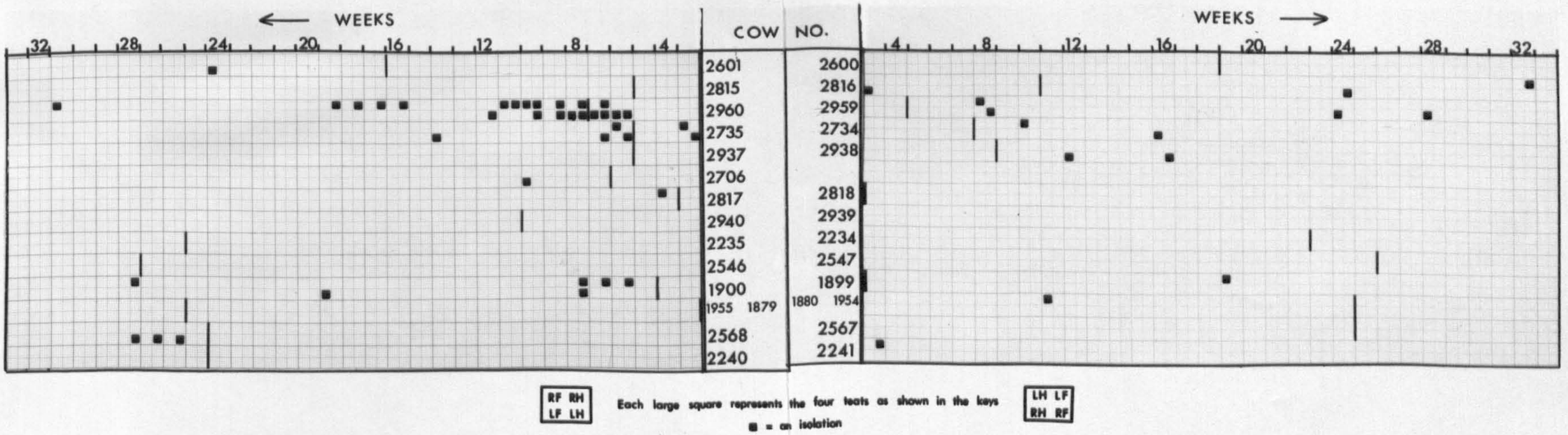


Fig. 13

Distribution of S. subgroup 3 (maltose +ve).

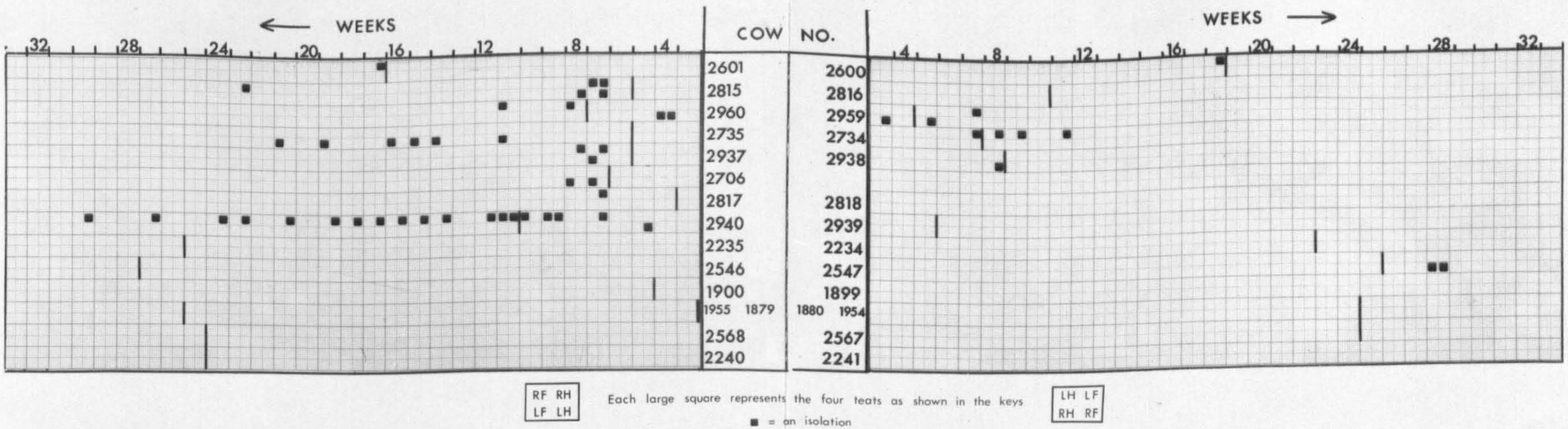


Fig.14

Distribution of S. subgroup 3 (Mannitol +ve)

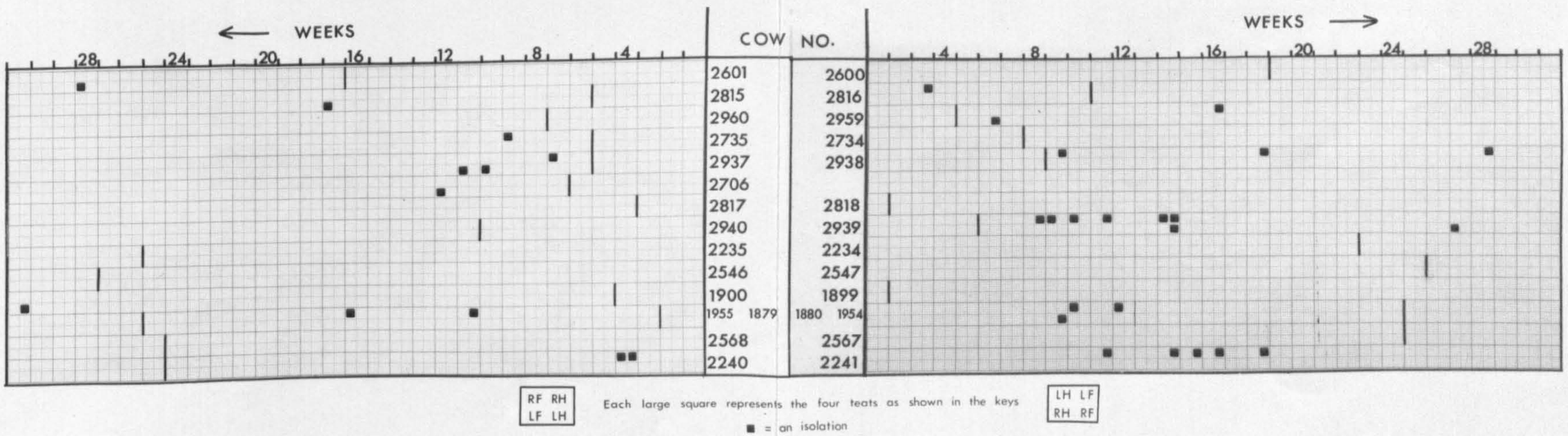


Fig. 15

Distribution of S. subgroup 6

her twin one week later (Figure 16). Subsequently it was isolated from cows above and below these animals suggesting a progressive spread from them.

S. subgroup 8 was isolated in week 10 from 1960 on the left side of the byre and then from 2938 on the right side in week 12 (Figure 17). Again there was evidence of progressive spread up and down the line of cows on each side.

The Micrococci

There were few isolations of cultures in M. subgroups 1, 2, 4 and 5 and their distribution will be discussed only in relation to their taxonomic similarities in a later passage.

The cultures in M. subgroup 3 were subdivided by the difference in the lactose reaction. The lactose negative cultures were too few in number for the epidemiological pattern to be assessed (Figure 18). Lactose positive cultures were isolated in the first week from eleven teat canals of seven widely separated cows (Figure 19). The incidence of infection remained high throughout the period although seldom persisting for long in any particular teat canal. They were widely distributed and no patterns of spread could be distinguished.

Micrococcus subgroup 6 cultures were divided according to the carbohydrates they were able to utilise. It will be seen that three strains were present in the herd at the start of the sampling period (Figures 21, 22 and 23 and their incidence diminished during the lactation.

The type which utilised all four carbohydrates is shown in figure 20.

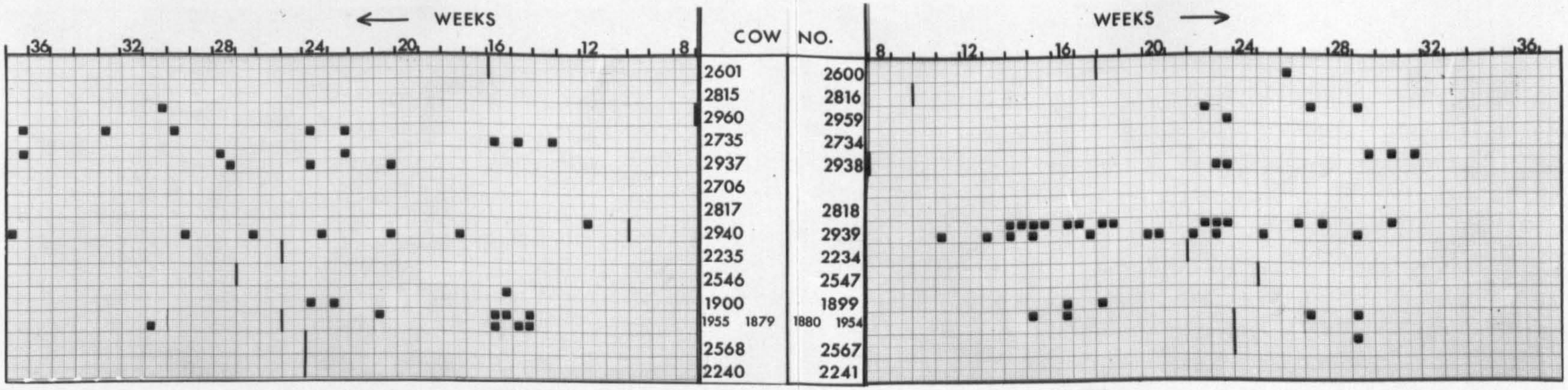


Fig. 16

Distribution S. subgroup 7

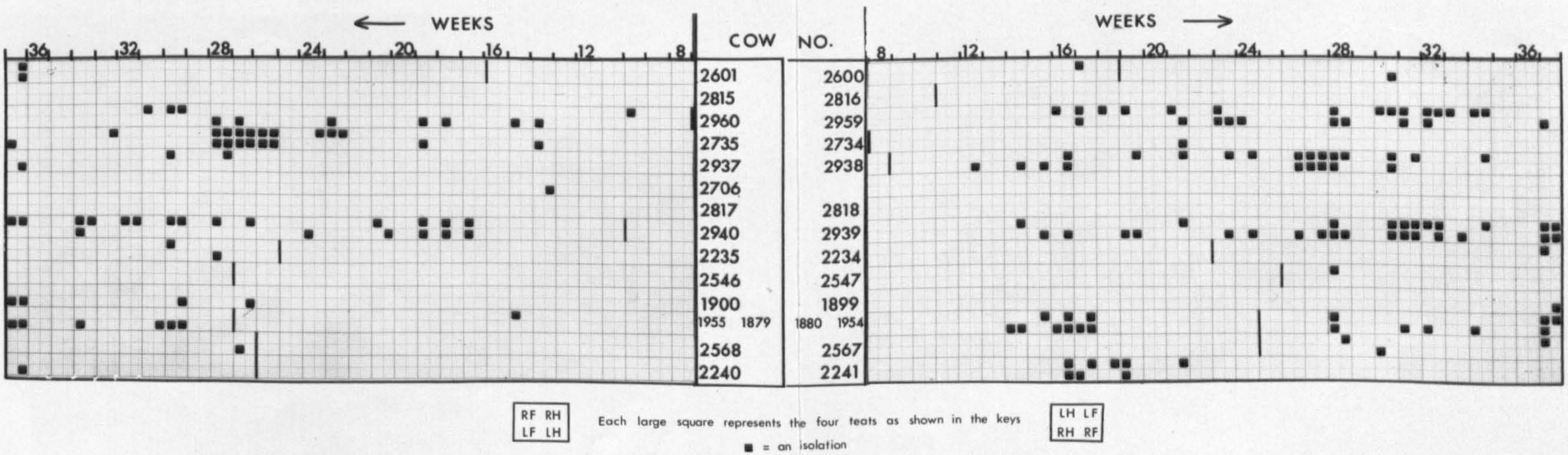


Fig. 17

Distribution of S. subgroup 8

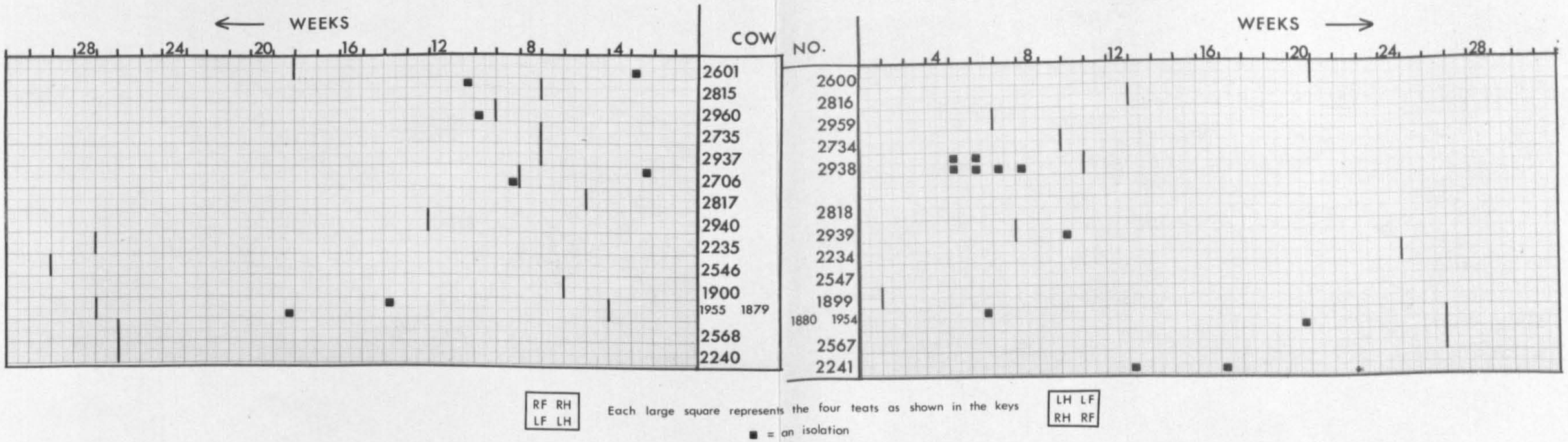


Fig. 18

Distribution of M. subgroup 3 (lactose -ve)

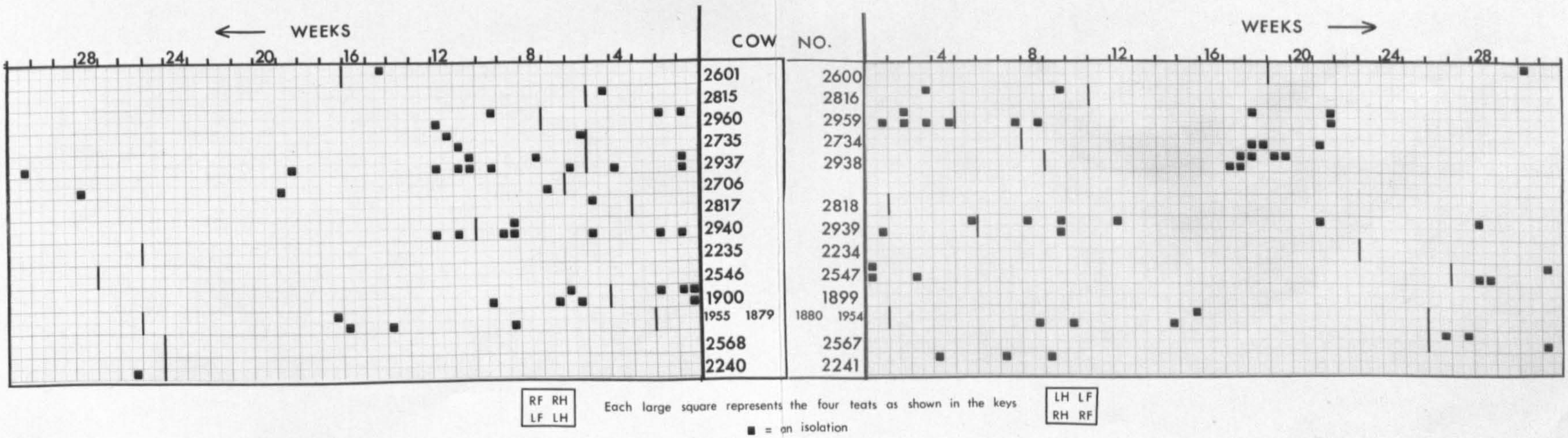


Fig. 19

Distribution of M. subgroup 3 (lactose +ve)

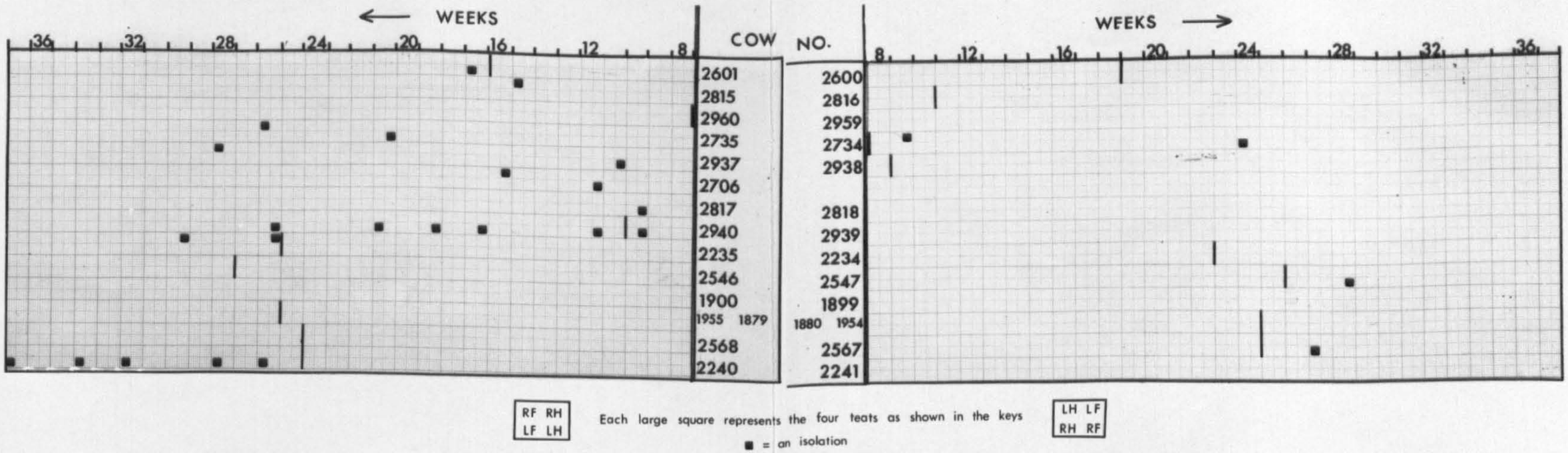


Fig. 20

Distribution of M. subgroup 6. (Arabinose, lactose, maltose and mannitol +ve).

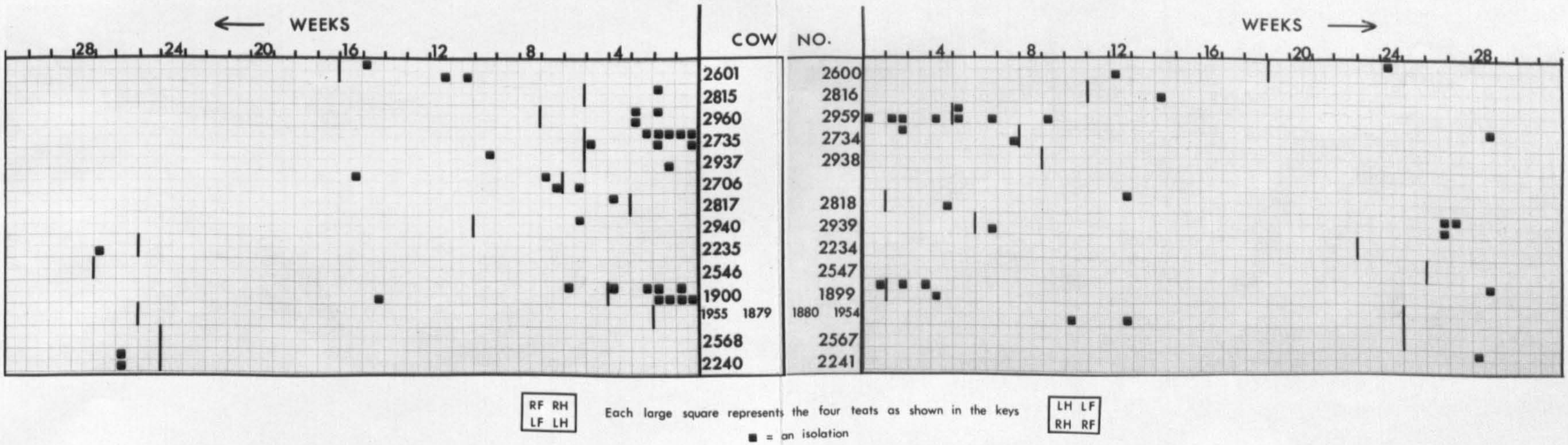


Fig.21

Distribution of M. 6 subgroup 6. (lactose +ve)

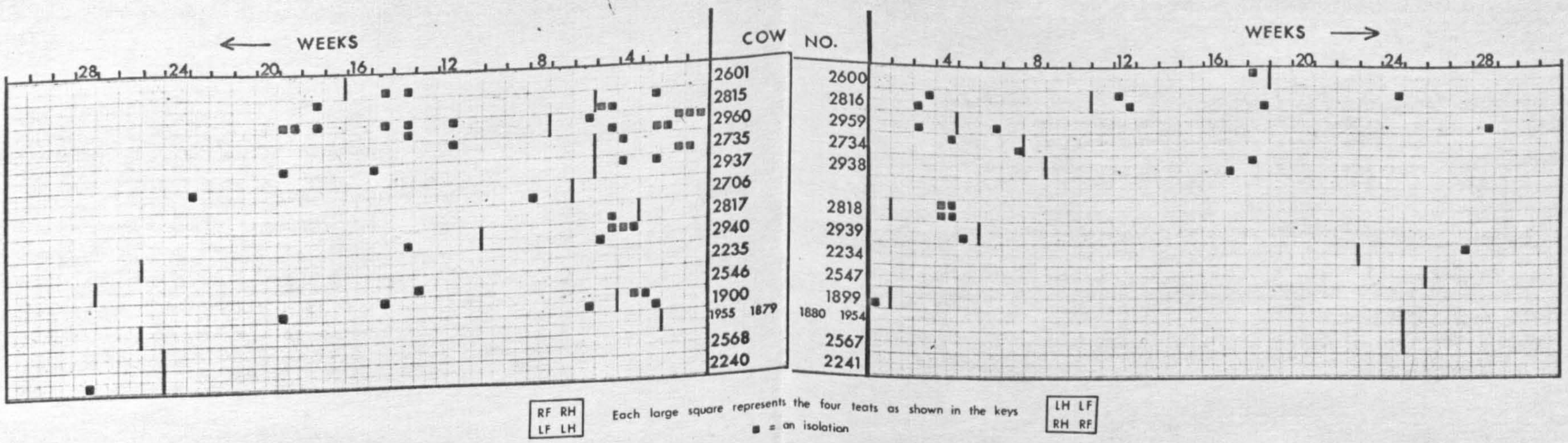


Fig. 22

Distribution of M. subgroup 6 (lactose and maltose positive: lactose maltose and mannitol +ve)

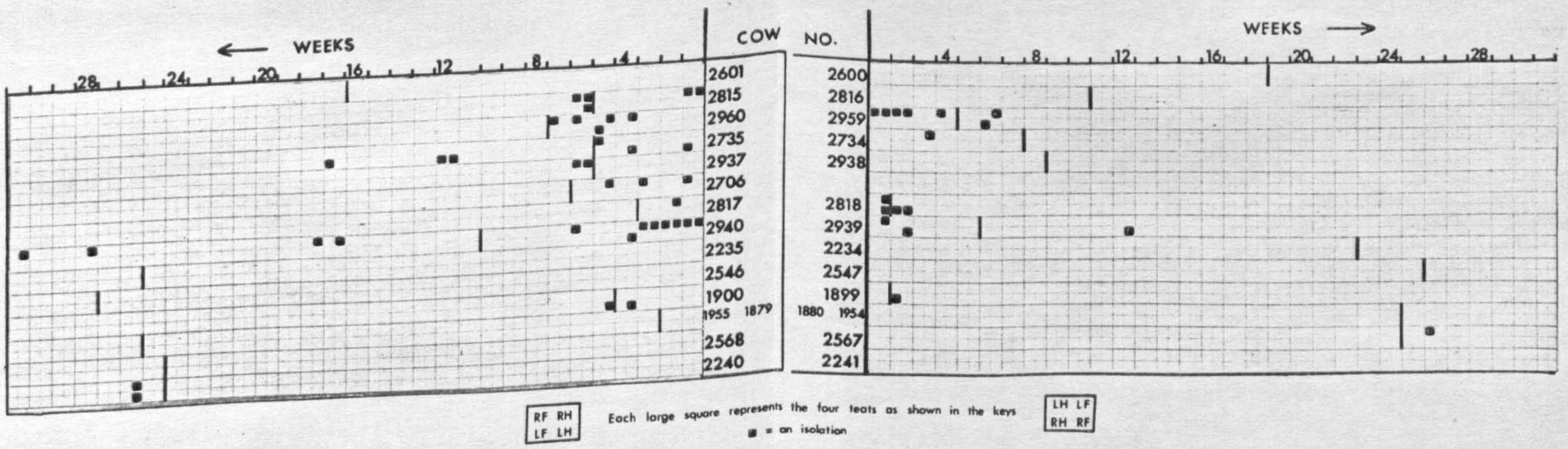


Fig. 23

Distribution of M. subgroup 6 (lactose and mannitol +ve)

It was first isolated in week 9 from 2734 and in week 10 from 2817 and 2940 on the other side of the byre. On this side it was isolated intermittently from the left hind teat of 2940 over a period of 16 weeks during which time it was also isolated from animals above and below her in the milking line.

Micrococcus subgroup 7

There were only 16 isolations of cultures of this group. The first two were in each of a pair of twins within one week of each other (Figure 24).

Micrococcus subgroup 9

The phosphatase and Voges-Proskauer positive cultures assigned to this group are shown in figures 25, 26 and 27. They were separated into 3 groups according to differing ability to oxidise carbohydrates. The type which oxidised all four carbohydrates shown in figure 25 was first isolated from 2601 in week 15 and from 2816 opposite and one standing below in the following week. From there it was isolated progressively down the byre. The distribution of the other lactose and maltose positive M. subgroup 9 cultures is shown in figure 26. There were too few isolations of this type for any conclusions to be reached regarding spread of the organism. The lactose, maltose and mannitol positive cultures in figure 27 show that the first isolations were at the bottom left hand side of the byre in week 3. In week 7 it was isolated from cow number 2600 at the top on the same side and also from 2815 on the opposite side and then appeared to progress slowly down that side.

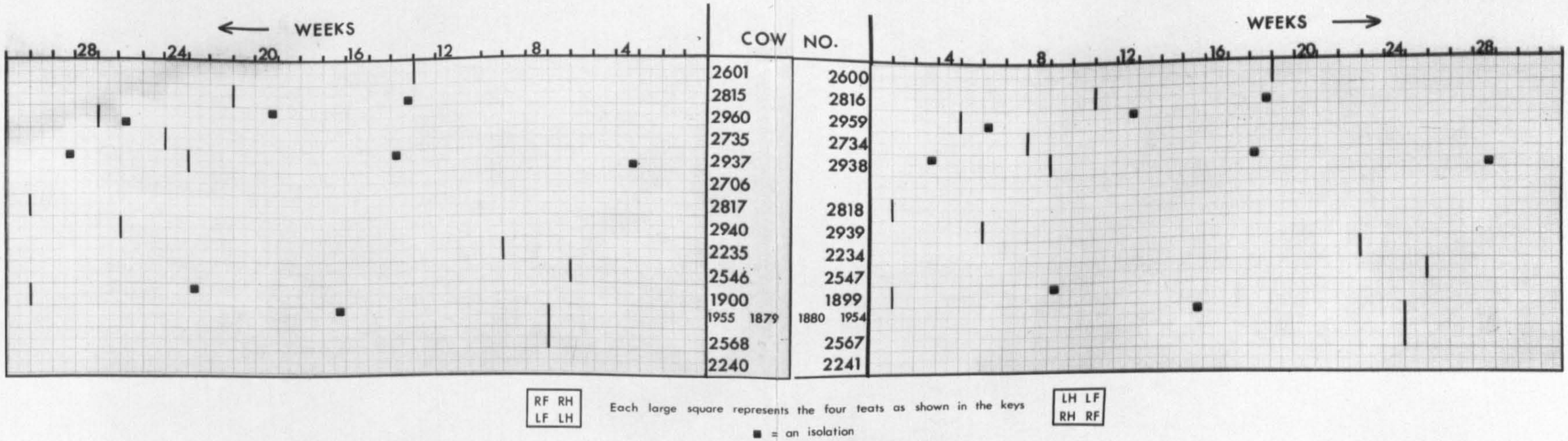


Fig. 24

Distribution of M. subgroup 7

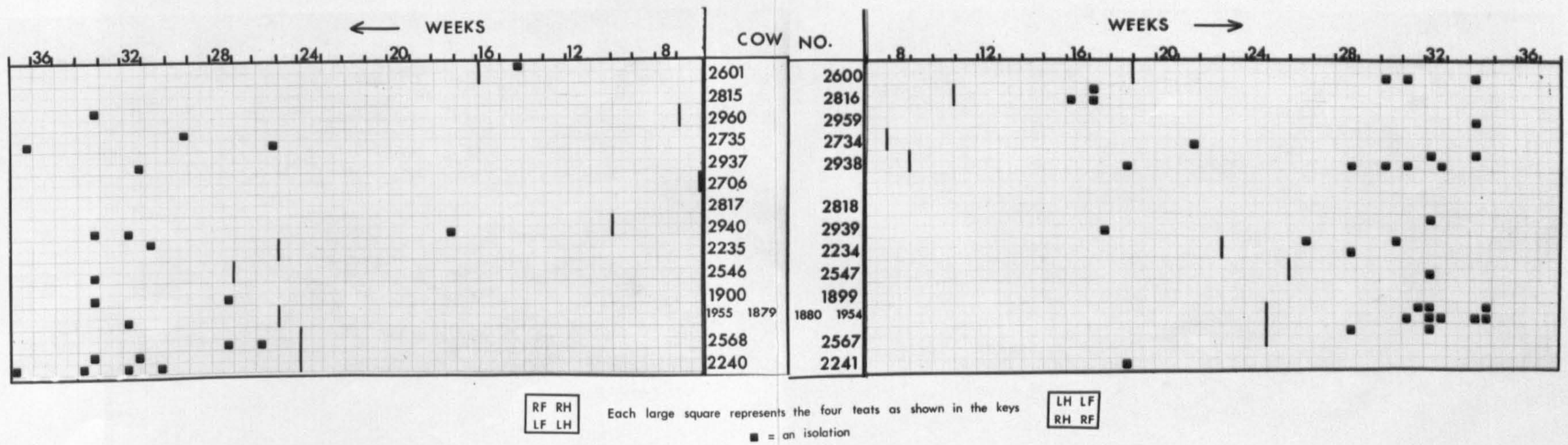


Fig. 25

Distribution of M. subgroup 9. (Arabinose, lactose, maltose and mannitol +ve).

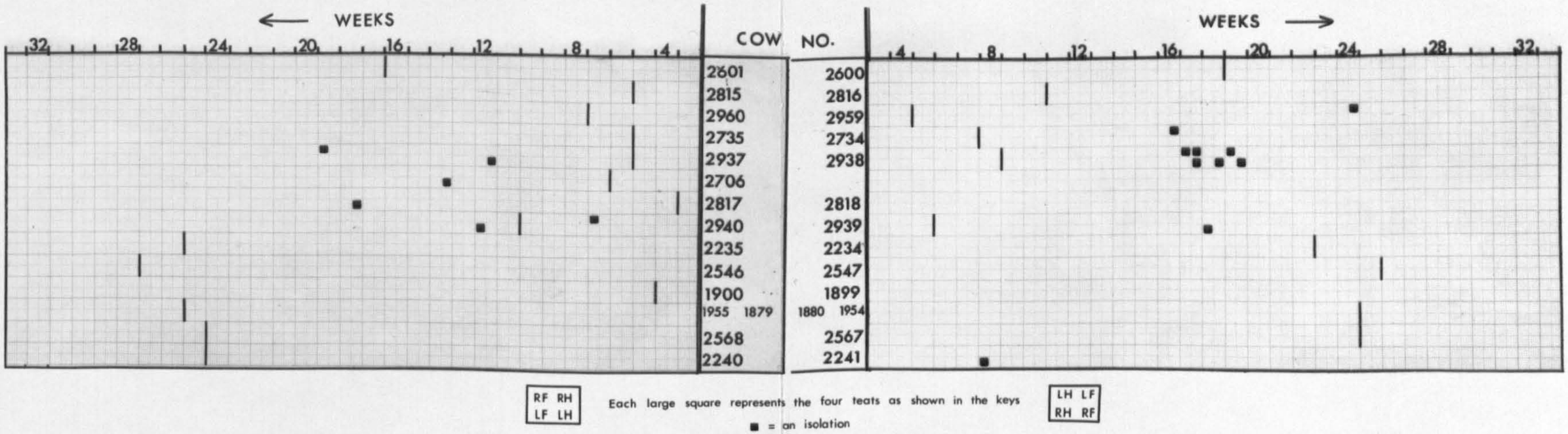


Fig. 26

Distribution of M. subgroup 9 (lactose and maltose +ve)

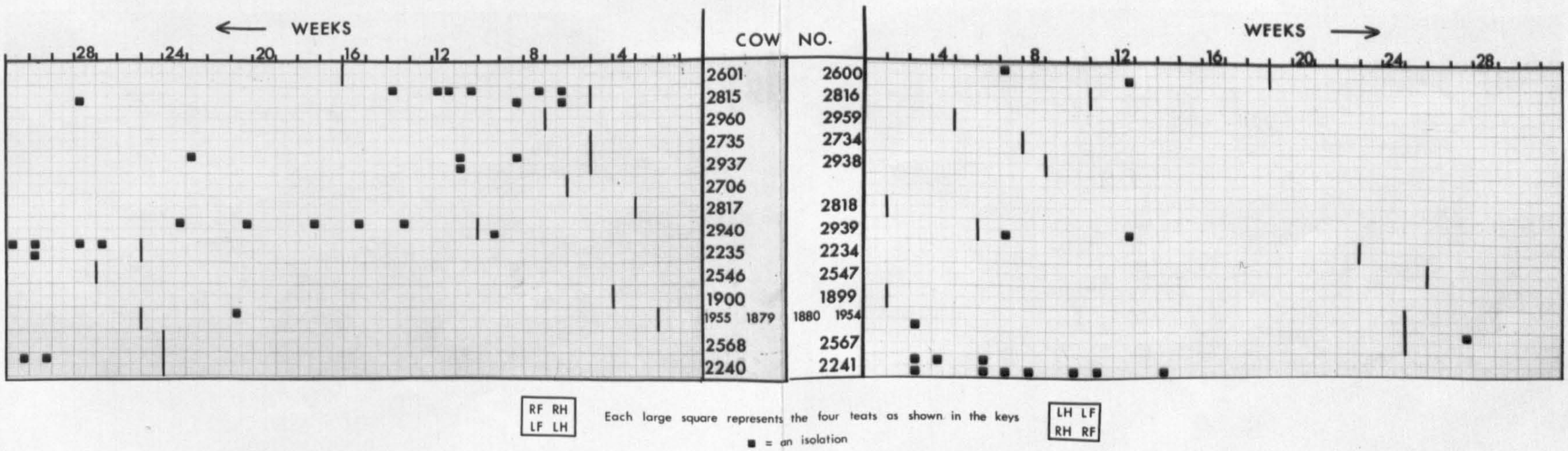


Fig. 27

Distribution of M. subgroup 9. (lactose, maltose and mannitol +ve).

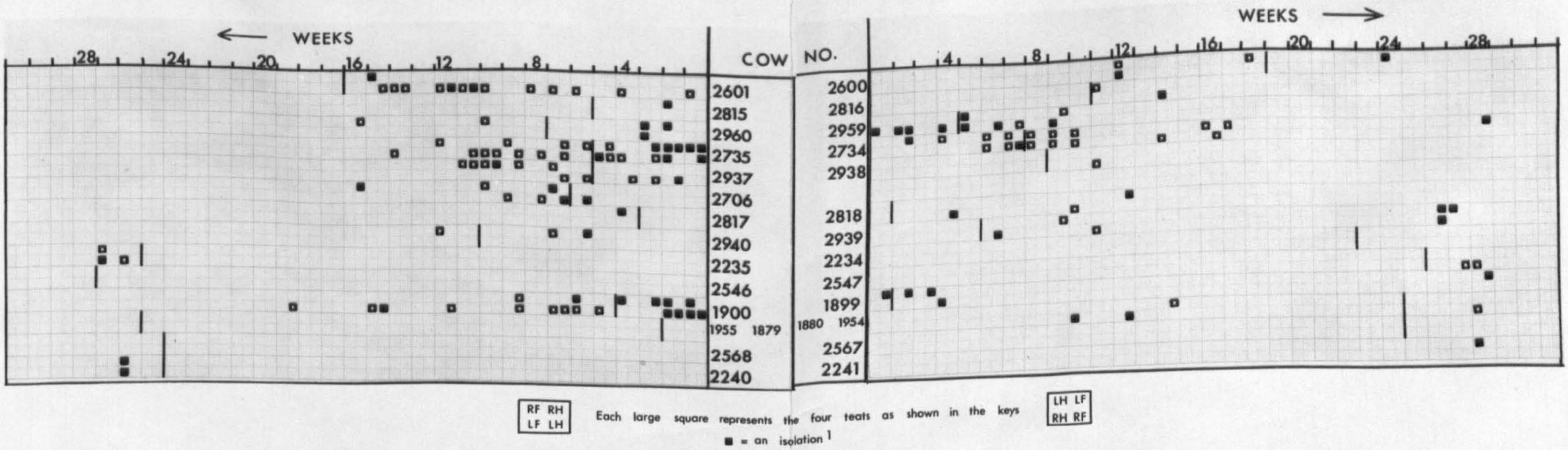


Fig. 28

Distribution of 1) M. subgroup 6 (lactose +ve)
and 2) S. subgroup 3.

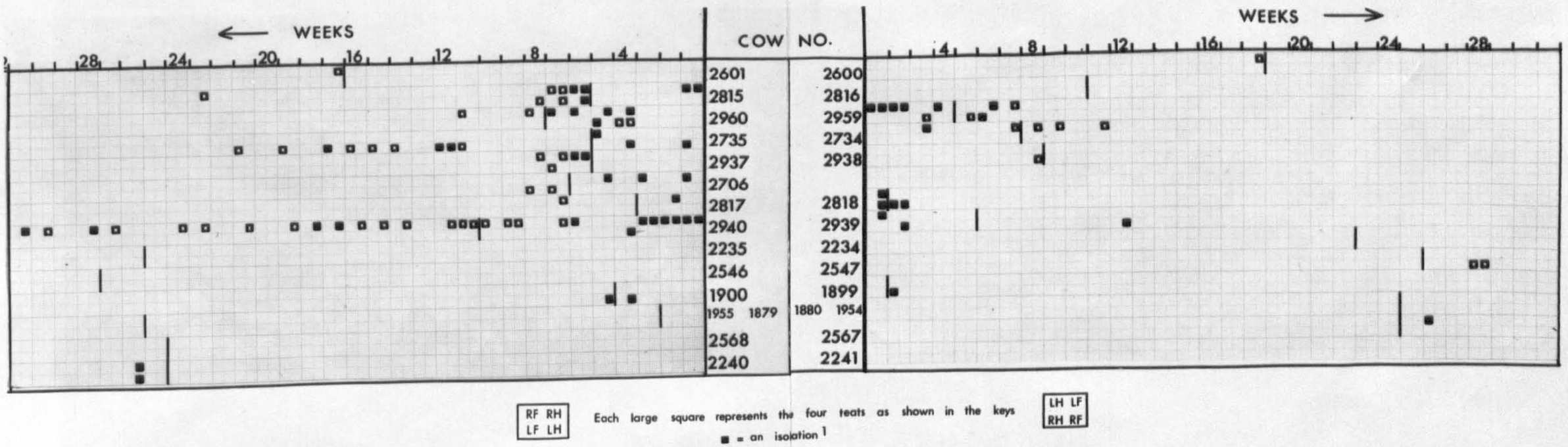


Fig. 29

Distribution of 1) M. subgroup 6 (lactose and mannitol +ve)
and 2) S. subgroup 3 (mannitol +ve)

The general patterns of spread of bacteria within the herd

The direction of spread on each side of the byre was assessed as having gone 1) in the direction of milking, 2) against the direction of milking and 3) in both directions. Spread across the byre was assessed as having gone 1) across in a forward direction 2) across in a backward direction or 3) directly across.

Spread on same side of the byre There was no significant difference in the frequency with which bacteria spread along or against the direction of milking. A majority of infectious spread in both directions.

Spread across the byre There was a tendency to spread across the byre in a forward direction but the numbers were not large enough for it to be significant. There was no ~~significant~~ obvious tendency for bacteria to spread from one to another in identical twins.

Epidemiological evidence for taxonomic similarity

In part V of this thesis the similarity of some atypical S. subgroup 3 cultures and M. subgroup 6 cultures was discussed. They were separable only by their ability to utilise glucose anaerobically but the test used to determine this did not give repeatable results. M. subgroup 9 cultures and other Micrococcus subgroups were also discussed. In order to assess any similarity between these groups of bacteria, unrelated subgroups with similar reactions were added to the same distribution map. This was intended to demonstrate if there was any continuity of infection between the subgroups.

Staphylococcus subgroup 3 and Micrococcus subgroup 6

The taxonomic results had shown that some S. subgroup 3 oxidised maltose or mannitol in addition to lactose. As these were to be compared to their counterparts in M. subgroup 6, typical lactose positive cultures of S. subgroup 3 were also compared. The distribution of the latter are shown in figure 28. There is evidence of similarity between the two subgroups in as much as the same cows tended to carry both types. The two types were also isolated intermittently from the same teat canal, for example the left hind of 2706. The similarity was more obvious however in the atypical S. subgroup 3 cultures which oxidised maltose or mannitol. Figure 29 shows those which utilised mannitol along with their counterpart M. subgroup 6 cultures. The apparent transition between cultures is marked. For example 2815 had the same 3 quarters infected with each type in successive weeks (Weeks 6 and 7). The two types interchanged in the left fore teat canal of 2735 while the right hind canal of 2940 had both types isolated from it intermittently throughout the period of sampling. The similarity was also evident between maltose positive S.subgroup 3 cultures and the equivalent M. subgroup 6, (figure 29A.) For example 2960 had both types intermittently in the right hind teat canal. It will be noticed however in weeks 18 and 19 both types were isolated. For this to have happened there must have been two cultures with different colonial or pigmental characters present on the primary isolation plate.

However, the epidemiological evidence strongly suggests a taxonomic similarity between S. subgroup 3 and M. subgroup 6.

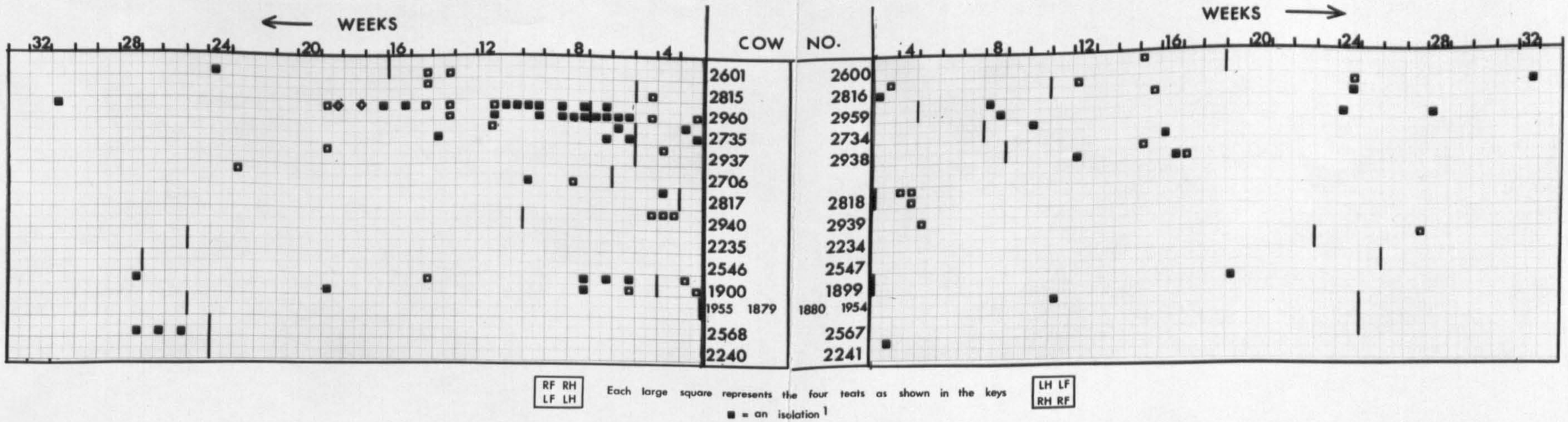


Fig. 29 A

Distribution of 1) M. subgroup 6 (lactose and maltose positive)

2) S. subgroup 3 (maltose positive)

and 3) Both cultures

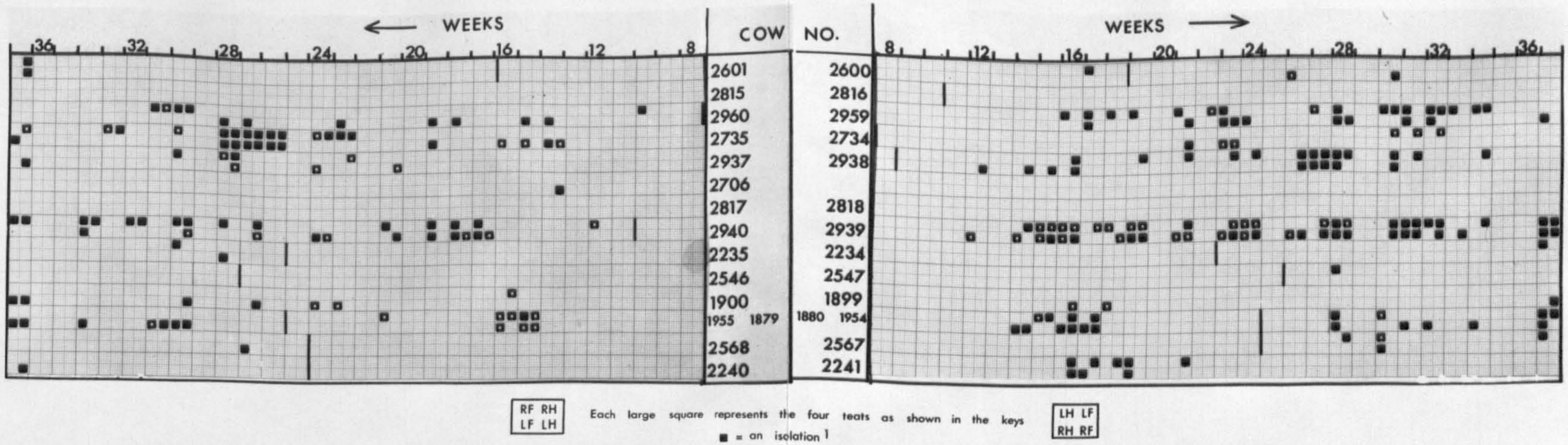


Fig. 30

Distribution of 1) S. subgroup 8
and 2) S. subgroup 7.

The atypical staphylococci

Two strains of staphylococci were isolated which were unusual in that they utilised arabinose. They differed only in their reaction to the phosphatase test and as their appearance in the herd was nearly coincident it was suspected that they were related. This is confirmed by their distribution which is shown in figure 30. The two types tended to be present in the same cows and were often isolated intermittently from the same teat canals.

Micrococcus subgroup 9

These cultures being phosphatase positive and Voges-Proskaeur positive were not typical of any subgroup described by Baird-Parker. However, as the phosphatase test used in this work was more sensitive than that used by Baird-Parker (1963) these cultures may belong to M. subgroups 1, 2, 3 or 4. There were too few isolations in M. subgroups 1 and 2 for any valid comparison to be made. M. subgroup 3 cultures were compared with their counterparts in M. subgroup 9 as shown in figure 32. The two types were not usually isolated intermittently from the same teat canal and are probably separate cultures. Similarly the arabinose, lactose, maltose and mannitol positive cultures in M. subgroup 4 are also probably different to their counterparts in M. subgroup 9. (Figure 31). On four occasions both types were isolated from the same teat canal in the same week and for this to have occurred there must have been a different character in the colonies on the primary isolation plate.

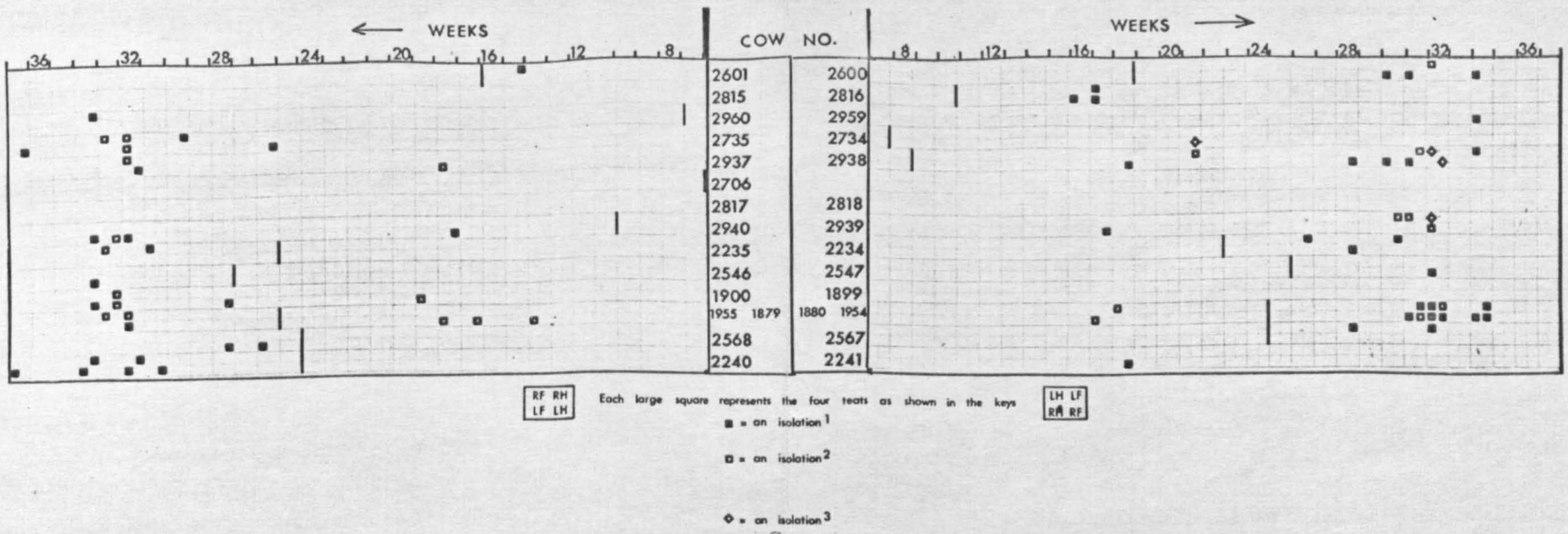


Fig. 31

Distribution of 1) M. subgroup 9 (arabinose, lactose, maltose and mannitol +ve)
2) M. subgroup 4
and 3) isolations of both cultures.

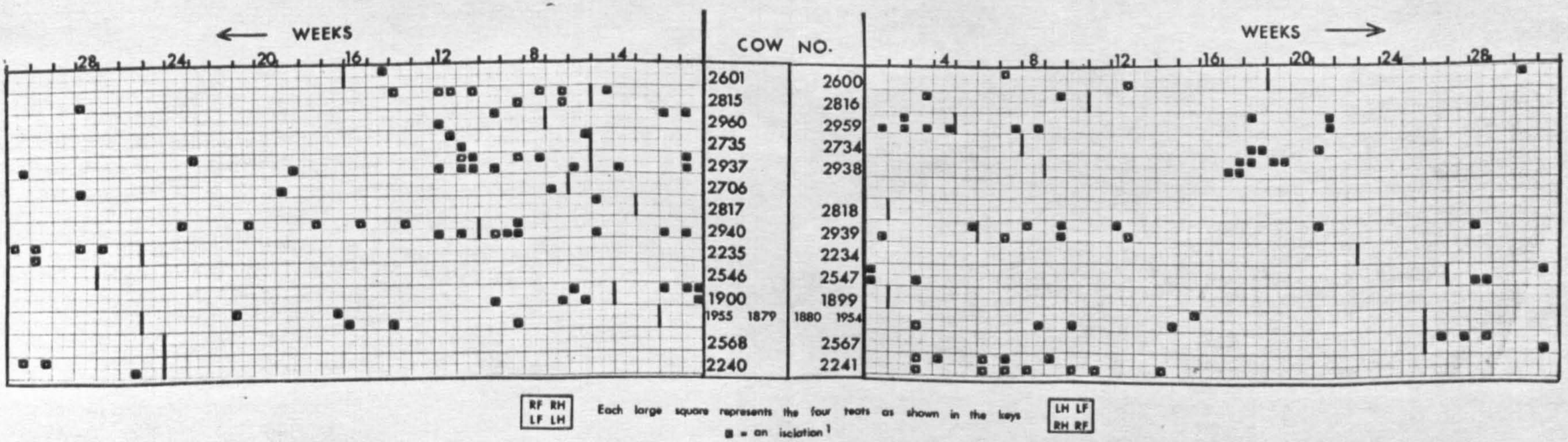


Fig. 32

Distribution of 1) M. subgroup 3 (lactose, maltose and mannitol +ve)
and 2) M. subgroup 9 (lactose, maltose and mannitol +ve)

The epidemiology of *Corynebacterium bovis*

The cultural methods used in this work were primarily for the identification of the *Micrococcaceae*. *C. bovis* did not grow well except when present in milk although pin point colonies were occasionally observed on plates prepared from broths inoculated with teat canal swabs. However, the presence of *C. bovis* on plates prepared with foremilk collected after teat canal swabs were taken has been analysed and the distribution of the bacterium in the herd shown in figure 33. The older cows at the bottom of the byre were already infected but of the heifers only number 2817 was infected at the start of the sampling period. During the following weeks only two animals in the herd remained uninfected and they stood at the top of the byre, (Numbers 2815 and 2601). Heifer 2601 did not have persistent infections but all the other heifers had the infection in all four teats at the end of the sampling period.

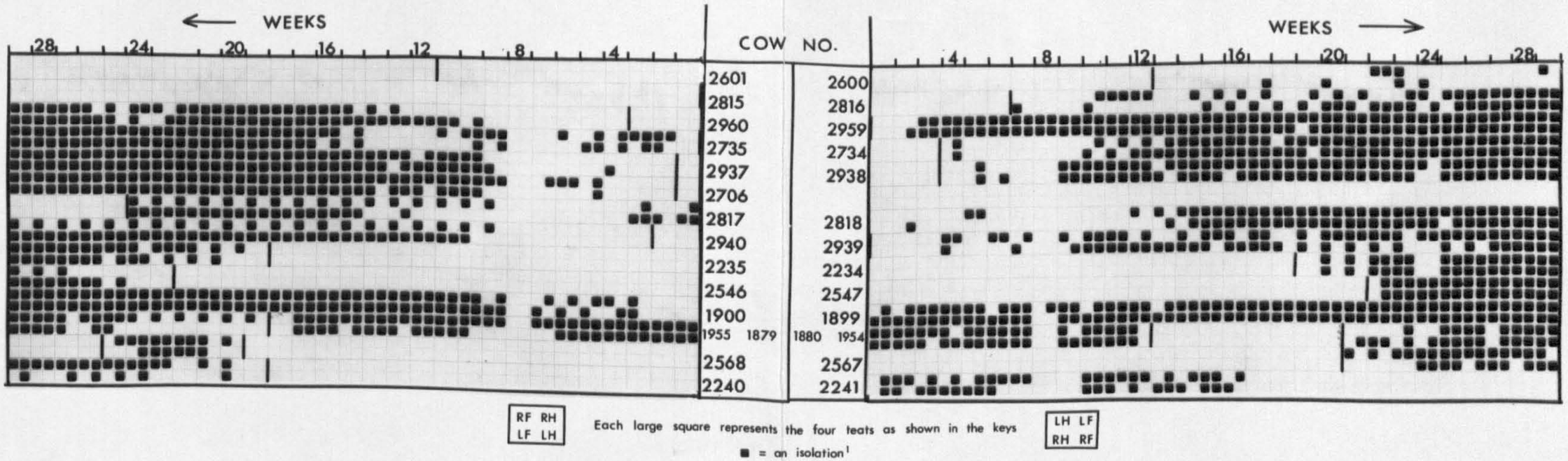


Fig. 33

Distribution of 1) Corynebacterium bovis

Discussion

The examination of infections present in bovine teat canals show the Micrococcaceae to be the common bacteria present there. The incidence of each of the subgroups fluctuated during the course of lactation.

The spread of the bacteria within the herd could be related to the cows' standing positions in the byre at milking time. All animals were at pasture throughout the day and were only brought in at milking time twice daily. While in the byre the animals would be closer than at other times and physical contact likely to occur. Consequently this would be the most probable time at which transfer of bacteria could occur. Most of the cows being twins tended to remain close to their partner while grazing, also being twins they are probably equally susceptible to the same infections. However, there was no marked similarity between the type of infections carried by each pair of twins and when bacteria spread across the byre there was no significant difference in the frequency it spread to the identical twin or to other animals. There was also no significant difference in the frequency in which bacteria spread up or down the milking lines in the byre. This suggests that even without the use of disinfectants the use of cold water flushed through the teat cups to reduce contamination is adequate in preventing direct transmission of bacteria between cows during milking. The spread across the byre, probably occurred when the cows were released after milking. Both rows of cows were released simultaneously and converged in the central passage to walk out the lower end of the byre.

This study was limited to the investigation of the bacteria resident at one specific skin site, i.e. the teat canal. Epidemiological patterns were discernible but it is highly improbable that the bacteria spread from one teat canal directly to another and similar patterns may have been evident from any area of skin. The results do suggest nevertheless that social contact between cows is an important method by which skin bacteria are transferred between animals.

The epidemiological evidence of taxonomical similarities tends to confirm the observation made in part V of this thesis that the anaerobic utilisation of glucose is not wholly satisfactory in separating the Staphylococcus and Micrococcus genera. This is particularly evident in cultures of S. subgroup 3 and M. subgroup 6. (Figures 27 to 29). Continuity of infection was apparent between types in these subgroups which shows that the other carbohydrate tests, phosphatase and Voges-Proskauer tests were all repeatable and the glucose reaction was not. On the other hand the phosphatase and Voges-Proskauer positive Micrococcus types appeared to be a separate group. This is supported by their ability to utilise dextrin, rhamnose and salicin as reported in an earlier section.

Part VII

The survival of Micrococcaceae in teat canal keratin

SUMMARY

Keratin was collected from 64 teat canals whose previous infective state was known. The samples were incubated for 60 days in a humid atmosphere. Micrococcaceae isolated from the keratin were identified by Baird-Parker's (1963) classification. All staphylococcal subgroups survived up to 27 days and some throughout the 60 day period. Micrococcal subgroups survived for at least 7 days and M subgroup 6 for 60 days. Bacteria were isolated from keratin samples after prolonged incubation when they had not previously been detected. The associated teat canals sometimes were found to have the same bacterium in vivo after keratin collection although the infection had not been suspected previously.

It is suggested that the physical characters of keratin enable it to retain bacteria without detection and that mechanical assistance is necessary for non-motile bacteria to permeate through the material.

Introduction

Adams and Rickard (1963) showed the fatty acids present in teat canal keratin to be inhibitory to Str. agalactiae. Hibbit and Cole (1968) showed the protein fraction of the material to be inhibitory to two strains of S. aureus and one Str. agalactiae strain. It has been argued in a previous section that the bacteria most commonly frequenting the udder parenchyma are those which are commonly present on skin. Teat canal keratin is essentially derived from the skin of the teat canal and the work reported here aimed to determine the longevity of survival of naturally occurring infections in keratin while incubated in vitro in conditions simulating the teat canal environment.

Materials and Methods

The sequence of sampling procedures for this experiment is shown in Table 3. The methods used for collecting milk samples and teat canal swabs is described in section IV. Keratin was collected using the technique of reaming the teat canal described by Murphy (1959) using Portex tubing.* The teat apex was first thoroughly cleaned with cotton wool soaked in methylated spirits. During collection the tubing was held in gloved hands which had been thoroughly washed and soaked in methylated spirits immediately beforehand. 3 cm. of tubing was taken from a 5 x 0.8 cm tube plugged with cotton wool with forceps, passed to the collector who then inserted it into the teat canal and removed a plug of keratin. The tubing and contents were then returned to the tube which was replugged and suspended in a sterile jar containing 2 ml. water closed with a screw cap and rubber washer.

---

*Portex polythene tubing. Bore 1.14 mm. Ext. dia. 1.57 mm.

The keratin was left in the tubing and incubated at 30°C in the jars throughout a nine week period. The presence of water in the jar ensured a humid atmosphere and avoided desiccation of the bacteria. At the intervals shown in Table 3 the tubing was removed using sterile forceps and an inoculum taken from the keratin using a straight platinum wire. This was then streaked onto a sheep blood (5 per cent) agar plate and incubated at 30°C for 48 hours. Colonies having the appearance of Micrococcaceae were subcultured and typed using the methods described in section V. Each colonial type present on the plates was examined.

Table 3

Plan of the experiment

Day -14. 1) Milk samples collected by syringe through the teat wall.

2) Hand drawn milk collected through the teat canal.

Day 0. Keratin collected from teat canals

Days 7 and 27. Sub-cultures taken from harvested keratin

Day 30. Samples as day minus 14.

Day 41. 1) Samples as day minus 14.

2) Sub-cultures taken from harvested keratin.

Day 60. Procedures as on day 41.

Results

Survival of infections in teat canal keratin in vitro

Keratin was collected from 64 teat canals and of these 16 (25 per cent) did not have Micrococcaceae isolated from them at all. The teat canals from which these samples were taken were uninfected when swabbed two weeks beforehand.

Staphylococci The types of staphylococci isolated from the remaining 48 samples during a 60 day period are shown in table 4 . Ten contained S. aureus during the period and of these nine were from teat canals known to have been infected before keratin collection, four of them with an associated intramammary infection. Three samples still had the infection at the end of the period and from one of these the organism had been isolated at each examination. S. subgroup 2 was isolated from two samples at the 27th day and was not subsequently re-isolated. In one sample S. subgroup 3 persisted throughout the period and this was from a quarter whose mammary parenchyma was also infected with the same strain.

S. subgroup 6 was isolated from only one keratin sample at the first examination and another sample at day 7. At the third examination at day 27 it was isolated from seventeen of the samples, in nine of which it was still present at day 41. In three it persisted to the last examination at day 60. S. subgroup 7 was isolated up to day 27 but not thereafter.

Number of keratin samples from which staphylococci were isolated

	Number of days incubation in vitro				
	Day 0	Day 7	Day 27	Day 41	Day 60
S. subgroup 1 (S. aureus)	6	10(6)*	10(9)	6 (6)	3 (3)
S. subgroup 2	0	1	2(1)	0	0
S. subgroup 3	2	2(2)	5(2)	3 (2)	1 (1)
S. subgroup 6	1	1	17	10 (9)	3 (2)
S. subgroup 7	5	0	2	0	0

* ( ) = No. of keratin samples from which the same bacterium was isolated at the preceding examination.

Table 4

The types of Micrococcus isolated from keratin samples is shown in table 5 . Only M. subgroup 6 persisted in any sample throughout 60 days. One isolation of M. subgroup 3 lasted to day 41 while M. subgroup 4 was only isolated at day 7 when it was present in the sixteen samples. M. subgroups 5 and 7 were isolated finally at day 27.

Comparisons of the concomitant infections in keratin and teat canals

The incidence of infections present in teat canals from which keratin was harvested is shown in tables 6 and 7. Table 6 shows the incidence of staphylococcal infections. S. aureus was isolated from eight teat canals two weeks before keratin collection, five of which were in quarters with intramammary infections. During the 60 days after collection two additional intramammary infections developed, one of which is especially interesting. This was in a quarter in which M. subgroup 5 was present as an intramammary infection at the start of the experiment. When the keratin was collected no bacteria were isolated but at days 7 and 27 both M. subgroup 5 and S. aureus was isolated from it. The latter had never been detected in this quarter previously. When milk was collected from the gland at day 30 M. subgroup 5 only was isolated and this was still in the udder parenchyma. At day 41 S. aureus was isolated again from the keratin and also from the teat canal. By the end of the experiment S. aureus was present as an intramammary infection while the micrococcus was not isolated from either the gland or the teat canal.

S. subgroup 2 was isolated from a keratin sample at days 7 and 27 and at day 30 the teat canal was also infected. This was the first time the

Numbers of keratin samples from which micrococci were isolated

	Number of days incubation in vitro				
	Day 0	Day 7	Day 27	Day 41	Day 60
M subgroup 3	3	4(1)*	0	1	0
M subgroup 4	0	16	0	0	0
M subgroup 5	3	3	1(1)	0	0
M subgroup 6	2	6(1)	5(3)	4(3)	2
M subgroup 7	0	0	1	0	0

*( ) = No. of keratin samples from which the same bacterium was isolated at the preceding examination.

Table 5

Numbers of Staphylococcal Infections present in teat canals in the period during which keratin was collected.

Staphylococcal subgroup	Before Day 0	Day 30	Day 41	After Day 60
S. subgroup 1 (S. aureus)	8(5)*	6(6)	12(6)	9(7)
S. subgroup 2	1	1	2	4
S. subgroup 3	3(2)	2(2)	4(2)	7(2)
S. subgroup 6	3	18(3)	17(3)	21(6)
S. subgroup 7	18(2)	1	0	0

*( ) = No. of quarters with the same infection present in the mammary parenchyma

Table 6

Numbers of Micrococcal infections present in teat canals in the period during which keratin was collected.

Micrococcal Subgroup	Before Day 0	Day 30	Day 41	After Day 60
M. subgroup 3	4(1)*	3	15	2
M. subgroup 4	4	0	0	0
M. subgroup 5	10	8(3)	2(1)	1(1)
M. subgroup 6	5(1)	2	9	6
M. subgroup 7	1	0	0	0
M. subgroup 9	1	0	0	0

*( ) = No. of quarters with the same infection present in the mammary parenchyma.

Table 7

subgroup had been found in this quarter. Although the infection did not persist in the keratin *in vitro*, at the end of the experiment this quarter and another of the same cow had intramammary infections with the same organism.

The most marked change was in the incidence of *S.* subgroup 6. Only three teat canals were known to be infected before keratin collection and after 30 days, the infection was isolated from eighteen teats; three of these were present as intramammary infections. There was a marked and rapid decrease in the incidence of *S.* subgroup 7 infections.

There was a general decrease in the incidence of *Micrococcus* infections in the period (Table 7) although at day 41 *M* subgroup 3 was isolated from fifteen teat canals.

The occasions when the same bacterium was isolated from teat canal keratin *in vitro* and the teat canal it was taken from at similar periods after collection is shown in table 8).

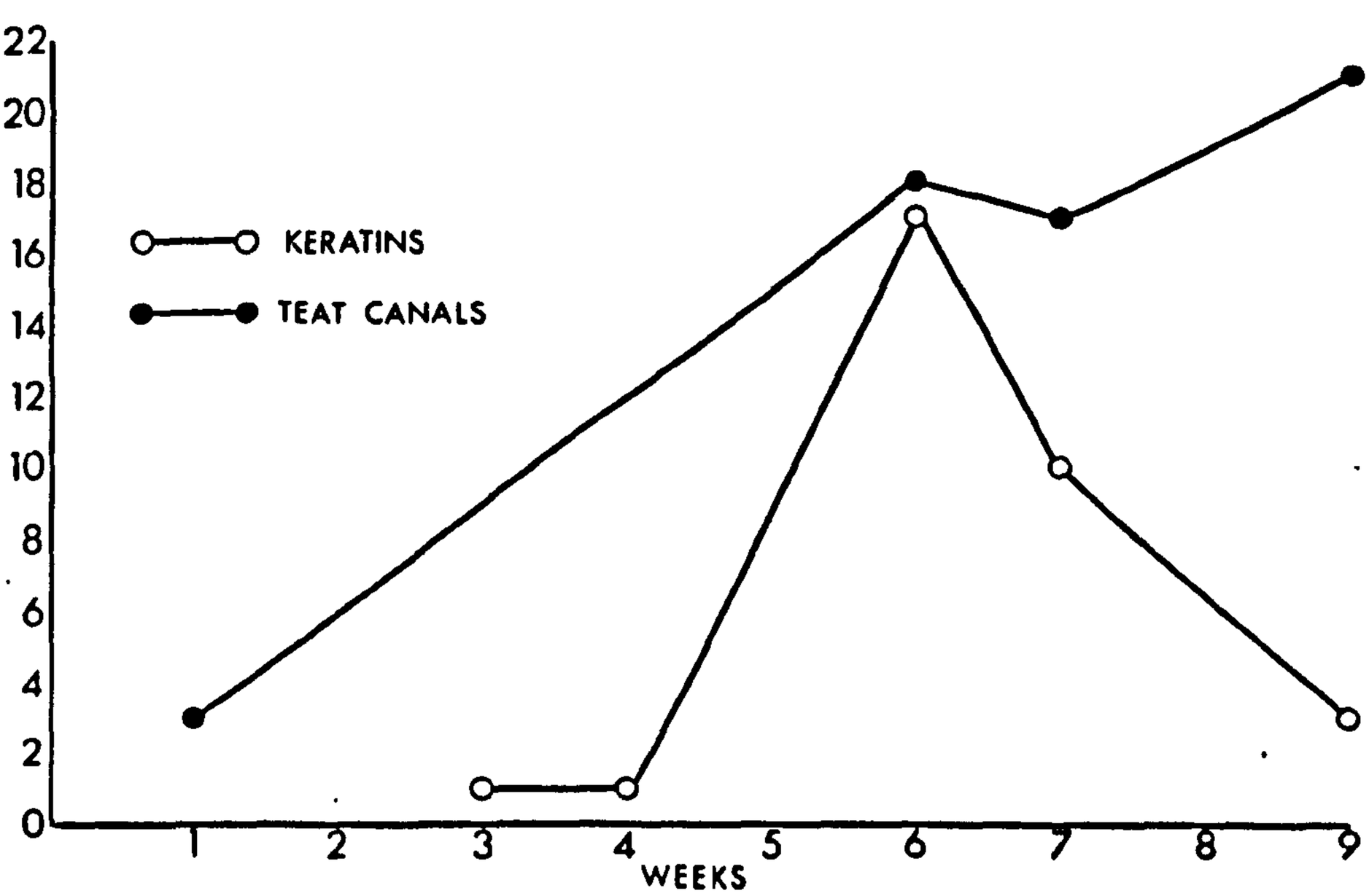
The observation of most interest is the manner in which *S.* subgroup 6 infections increased in incidence *in vivo* and *in vitro*: this is shown in figure 34 . Of seventeen keratin samples infected 27 days after collection 10 were from teat canals also found to be infected at the same time. The bacterium had been detected in only two separate keratin samples in the first two examinations and only three teat canals were known to be infected before keratin collection.

At 6 weeks 21 teat canals had *S.* subgroup 6 isolated from them, not all of which had been infected at 41 days. In fact the organism was isolated from 33 separate teat canals during the course of this experiment.

Numbers of infections common to both keratin *in vitro* and the teat canals from which it was collected.

	Staphylococcal Subgroups				Micrococcal Subgroups	
	1 (*S.aureus*)	2	3	6	5	6
Day 27/30	6	1	2	10	1	1
Day 41	5	0	2	5	1	4
Day 60	2	0	1	2	0	1

Table 8



Incidence of S. subgroup 6 infections in teat canals and keratin samples.

Fig. 34

Micrococcus subgroup 5 and 6 were the only ones to be found in keratin and the associated teat canal. The M subgroup 5 has already been described. M. subgroup 6 was common in four cases to both keratin and the teat canal at day 41, and in one of these it was the first time it had been isolated from the keratin in vitro. It was detected in the teat canal for the first time at day 30. In the other cases the bacterium had previously been detected in keratin samples and in the teat canals before collection.

Discussion

This experiment aimed to find out how long bacteria present in teat canal keratin survived in conditions which simulated the natural state. Micrococcaceae were classified by Baird-Parker's (1963) methods and of the subgroups isolated M. subgroup 4 was not recovered from keratin after 7 days. All other subgroups survived to 27 days while S. aureus, S. subgroups 3 and 6 and M subgroup 6 survived throughout 60 days. The method used for culturing the keratin did not always detect bacteria susbequently shown to be present. This is probably because the physical character of the material will not enable bacteria to permeate through it without mechanical assistance which was effected *in vitro* by the method of culturing bacteria from the keratin. This may also be true for keratin in *situ* in the teat canal since S. aureus was isolated at days 7 and 27 in a keratin sample from a teat canal not known to be infected previously. When samples were taken at day 30 from the teat canal S. aureus was again not detected although when subsequently sampled the same S. aureus strain was found and at the end of the experiment had caused an intra-mammary infection. Similarly S. subgroup 2 was isolated in one keratin sample and S. subgroup 6 in ten samples 27 days after collection from teats which 30 days after collection were found to contain the same infections where they had not previously been detected. Since the keratin samples were shown to be infected the infections must have been present in the teat canals at the time of keratin collection. The ability of keratin to retain infections supports observation of the author that teat canal infections are more easily recognised by

a swab inserted into the canal than by simply drawing milk through the canal. The practice of discarding the first streams of milk is of little use for rather than flushing bacteria out of the canal it does not cause sufficient agitation to mobilise bacteria in it.

Adams and Rickard (1963) showed keratins from different quarters had varying degrees of inhibition against Str. agalactiae. In the work reported here in which natural infections were used no such variation could be determined. In fact it is possible that the infections were from teat canals susceptible to that particular type. Nevertheless the duration which the bacteria are able to survive is important as they may provide a reservoir of infection capable of infecting the mammary parenchyma. This is supported by previous work (Forbes ~~et al~~ and Hebert 1968) which showed that some teat canals were infected for long periods before the bacteria reached the teat sinus.

Part VIII

Studies in the pathogenesis of mastitis

SUMMARY

Intramammary infections in all quarters of 18 cows was determined by collecting milk samples by syringe from the teat sinus. These samples were taken within one week before calving, eight weeks after calving and thereafter at monthly intervals throughout lactation. The role of teat canal infections in bacterial invasion of the udder parenchyma is described.

Cell counts of foremilk taken each week were made with an electronic counter (Cullen 1967). All intramammary infections are reported and the type related to the mean cell count levels. Corynebacterium bovis infected all but one quarter, often concurrently with other infections. C. bovis and Staphylococcus epidermidis caused significantly higher cell counts than those in uninfected quarters but did not cause clinical mastitis which was only associated with Staphylococcus aureus and Streptococcus uberis. S. aureus caused the highest cell counts throughout lactation. The significance of high cell counts in quarters infected with relatively non-pathogenic bacteria is discussed in relation to the susceptibility to infection by other pathogens.

INTRODUCTION

It is known from the work of Schalm and his co-workers (see below) that a pre-existing leucocytosis in milk within the mammary gland protects the gland from experimentally induced infections with pathogenic bacteria. In their experiments a leucocytosis was induced by intramammary inoculations with saline or other solutions injurious to the milk secreting tissue but Schalm, Lasmanis and Carrol (1964b) reported that a mild inflammatory reaction due to infection with Streptococcus uberis inhibited multiplication of 500,000 Aerobacter aerogens introduced into the gland.

In a later paper Schalm ~~et al~~ Lasmanis and Carroll, (1967) found that when Streptococcus agalactiae was inoculated into mammary glands multiplication of the bacterium was sometimes delayed but was not associated with the infiltration of large numbers of neutrophil leucocytes throughout the period. They proposed a hypothesis that cross reacting streptococcal antibodies may exist and suppress Str. agalactiae in udders already infected with other streptococcal species. Jain, Lasmanis and Schalm (1967) showed that an induced leucopenia allowed A. aerogenes and a coagulase-negative staphylococcus to multiply rapidly. The latter bacterium then caused an actue mastitis within 24 hours, which was controlled by a subsequent massive infiltration of neutrophils. Edwards and Jones (1966) reported that the number of white cells in the foremilk of cows which contained coagulase-negative staphylococci did not exceed 1×10^6 cells/ml. Nevertheless these quarters were not

easily infected with S. aureus even though the bacterium was occasionally isolated from their milk. The authors thought that the coagulase-negative bacteria populated the teat duct, an environment considered more favourable to these types than to S. aureus, and that the leucocyte level was not a factor in preventing the latter from establishing intramammary infections. However, Schalm ~~et al~~, Lasmanis and Carroll (1966) showed that 200,000 to 500,000 cells/ml protected quarters from experimental streptococcal inoculations, a level of cell count found by Forbes and Hebert (1968) to be associated with intramammary infections of coagulase negative staphylococci. In investigating the mechanism by which bacteria invade the udder parenchyma, it is therefore important to know the effect non-pathogenic bacteria have on the cell count of milk. This paper reports the cell count levels of foremilk associated with naturally occurring intramammary infections during one lactation of an experimental dairy herd. The role of teat canal infections in bacterial invasion of the udder parenchyma is described. The pathogenic effects of various intramammary infections is related to the susceptibility of the gland to invasion by more virulent bacteria.

MATERIALS AND METHODS

The animals used in this work are shown in figure 4 and described in part IV. Milk samples and teat canal swabs were taken weekly as described in part V.

Intramammary infections Milk samples were collected by syringe through the teat wall used the method described earlier (part IV). These samples were collected when possible during the week before calving, eight weeks after calving and thereafter at monthly intervals. Some colostral samples were too viscous for syringe collection and later samples were occasionally missed because of difficulty in collection. Milk collected by this means was sown on five separate plates (0.1 ml per plate) for primary isolation. Micrococceae from all samples were identified as described in part V. Corynebacterium bovis was recognised by colonial and morphological characters.

Analysis of cell counts Milk cell counts from quarters with the same intrammary infections and at the same period of lactation were grouped together. Values were transformed to logarithms and the geometric means determined. Because of varying lengths of lactations in different cows, the start, middle and end of each lactation was compared. The cell counts from the first and last three months of lactations were grouped together for comparison leaving an intermediate period of varying duration. The week numbers of the lactation are numbered progressively forward to the intermediate period and progressively backward from the end of lactation after the intermediate period. Those latter week numbers are denoted by a minus sign before the number.

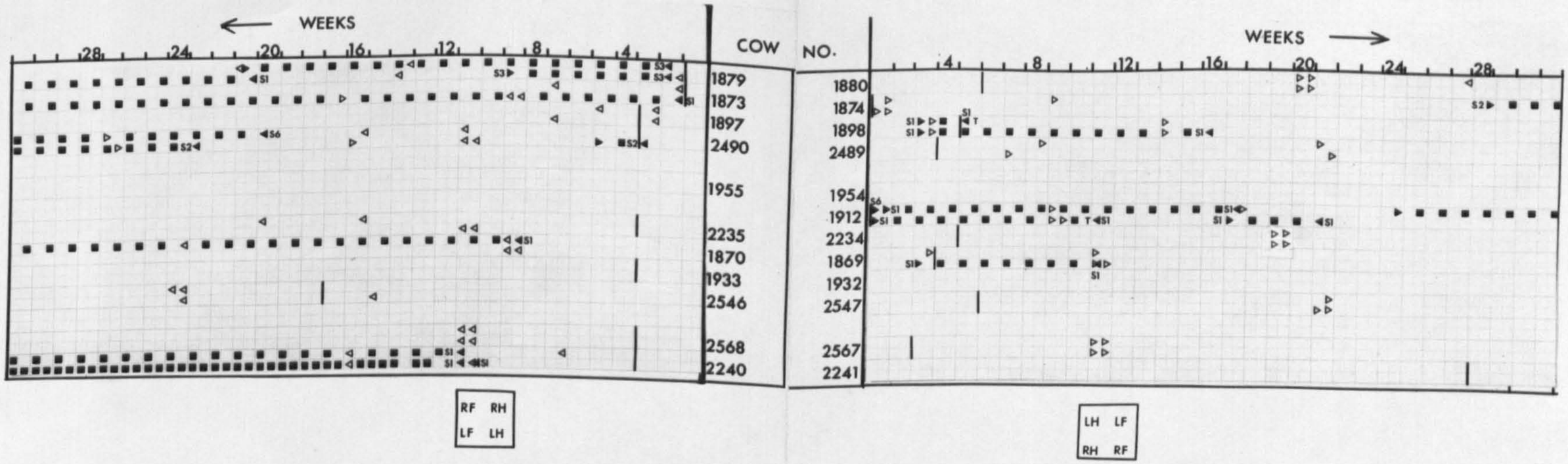
RESULTS

The role of teat canal infections in bacterial invasion of the mammary gland

The types of intramammary bacteria ~~present~~ confirmed to be within the udder by being present in milk taken by syringe from the teat sinus are shown in figure 35. The times at which the infection was first isolated and finally isolated in milk collected by syringe is shown by triangular symbols. - Micrococcaceae infections are shown as continuous infections by the relevant quarters of the graph paper being filled by closed squares. Because C. bovis infections were so frequent, only the start and where appropriate, the termination of the infections are shown both by open triangles.

C. bovis During the lactation all quarters of the cows with the exception of the left hind of number 2547 had intramammary infections with C. bovis, The occasions when the bacterium was isolated in normally drawn milk are presented in figure 36 in the same manner as described for figures in section VI.

C. bovis did not grow as identifiable colonies from teat canal swabs but evidence for the infection being present solely within teat canals was found in differences between normal hand drawn milk and that collected by syringe. The infection was at times found to be in milk drawn through the teat canal but absent in milk taken by syringe from the teat sinus. A majority of the quarters had intramammary infections at the start of lactation or were found to be infected when milk samples were taken by teat wall puncture after eight weeks. However, in ten quarters milk



▷◁ = Corynebacterium bovis infections

▶■◀= Micrococcaceae infections (See below)

T = Clinical mastitis treated by antibiotic therapy.

S1 = S. aureus

S2 = S. subgroup 2

S3 = S. subgroup 3

S6 = S subgroup 6.

The symbols described above show the first and where appropriate the last occasions when the relevant bacterium was present in milk collected by syringe from the teat sinus through the teat wall.

Minimum duration of intramammary infections

Fig. 35

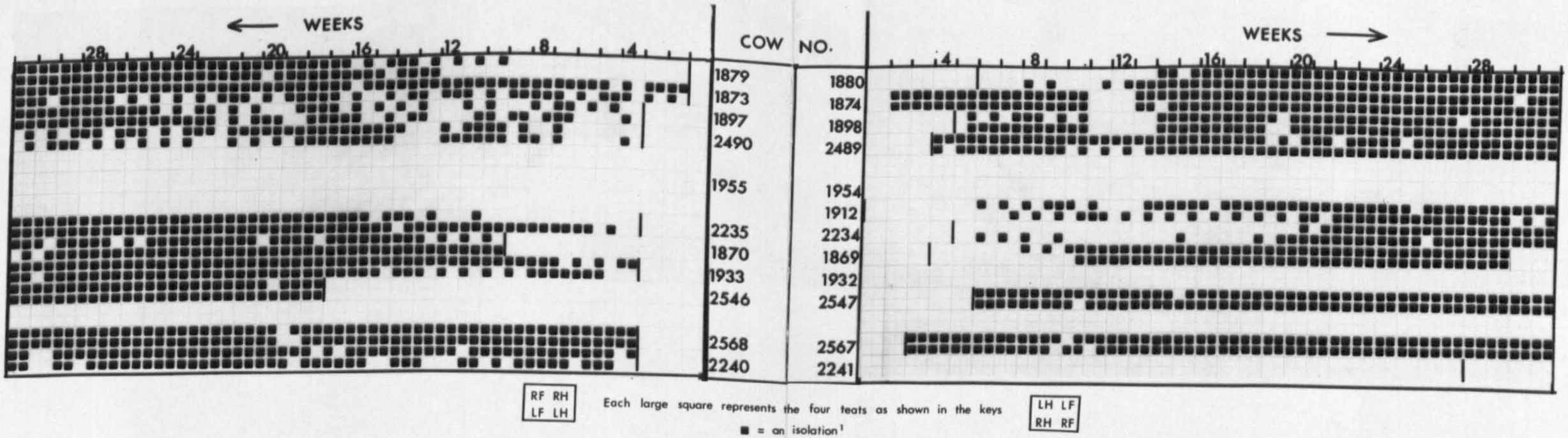


Fig. 36

Distribution of 1) Corynebacterium bovis

from the teat sinuses were uninfected while milk drawn through the teat canal had C. bovis present. One of these quarters had two sterile teat wall puncture samples taken at an interval of one month. Subsequent samples from all quarters were infected. These results show that C. bovis present in the teat canals preceded invasion of the udder parenchyma. In one quarter the duration of the teat canal infection was greater than 4 weeks.

S. aureus caused intramammary infections in 11 quarters (figure 35) and it was widely distributed in other teat canals (Figures 37 and 38). One of the three types of S. aureus present in the herd was isolated from eighteen teat canals over periods of at least three weeks from quarters that never became infected.

Seven quarters had intramammary infections persisting from the previous lactation. The left hind quarter of cow 1879 had a teat canal infection at the start of the lactation and an intramammary infection was not confirmed until the 20th week. During the preceding weeks milk samples taken through the teat wall did not contain the bacterium. By these results it was confirmed that the intramammary infection developed between the 16th and 20th week. The three quarters of cow 2240 which had intramammary infections may have been infected at calving but samples could not be taken until the 9th week of lactation because of the extreme turgidity of the udder and the conformation of the teats.

Coagulase negative staphylococci S. subgroups 2, 3 and 6 were the only coagulase negative types to cause intramammary infections in this

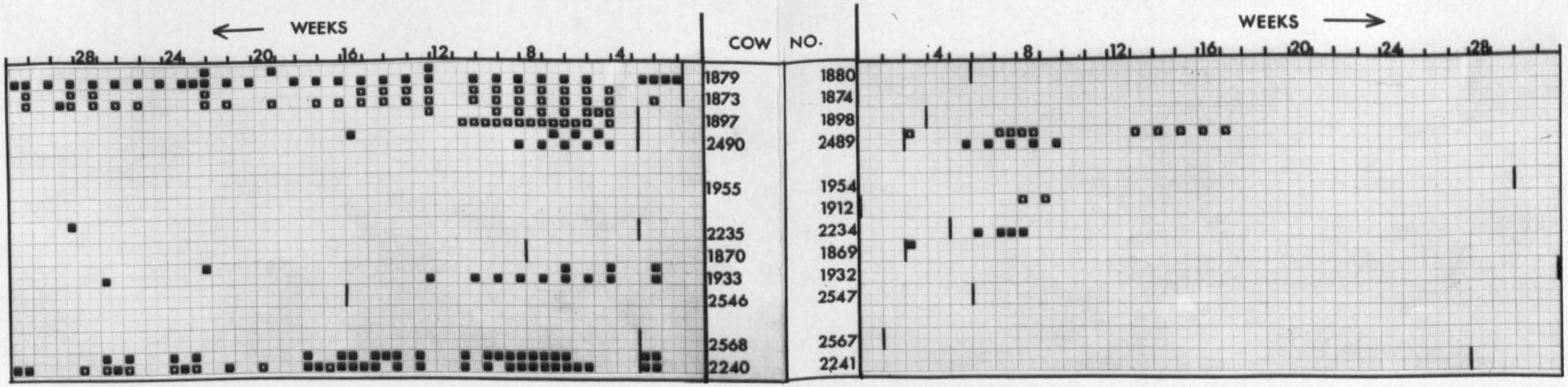


Fig. 37

Distribution of 1) S. aureus (Phage type 29/52/42E/101/110)

and 2) S. aureus (Phage type 29/52/42E/101/110/53/42D +).

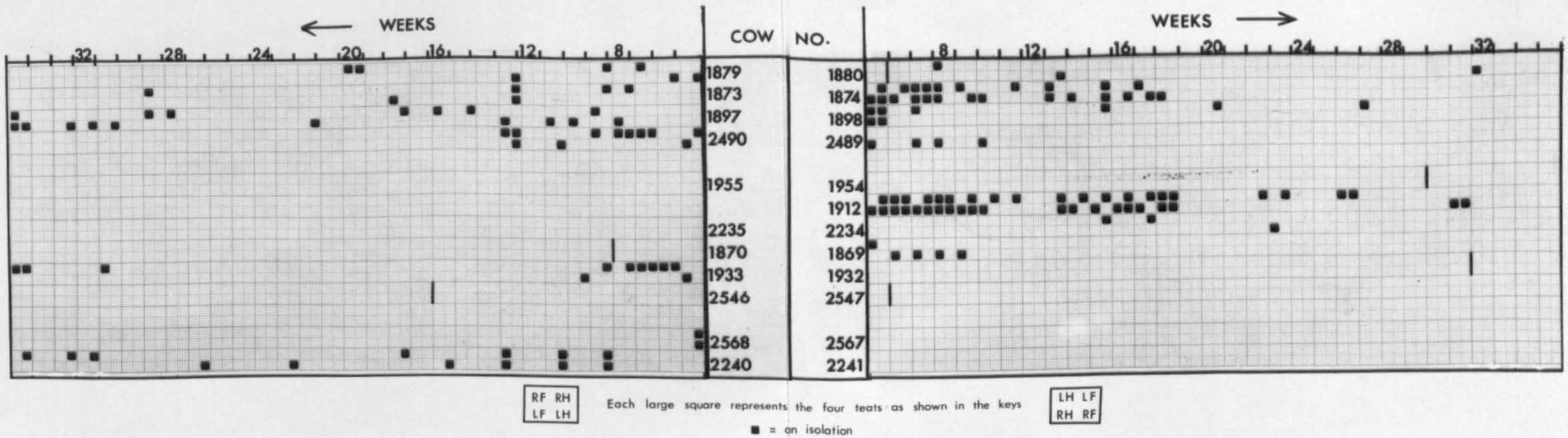


Fig. 38

Distribution of 1) S. aureus (Phage type - untypable)

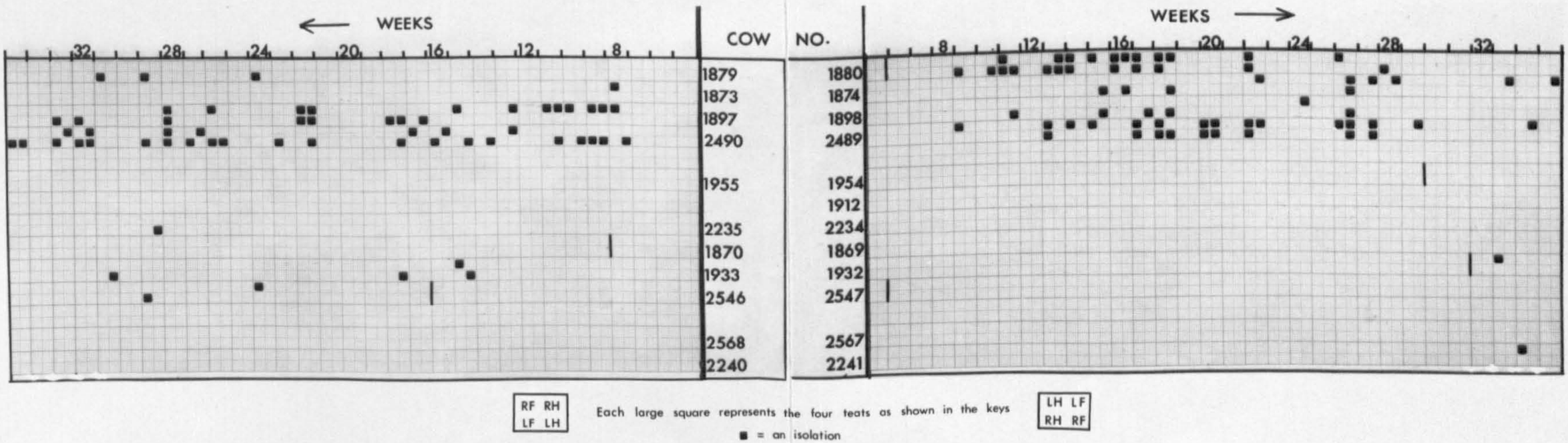


Fig.39

Distribution of 1) S. subgroup 2.

lactation. The distribution of S. subgroup 3 was mainly confined to cow number 1879 which had two quarters with intramammary infections. The distribution of S. subgroups 2 and 6 are shown in figures 39 and 40 respectively. It can be seen that both types were common in teat canals and by comparing the figures with figure 35 it will be observed that in those quarters with intramammary infections the bacteria were isolated from the teat canals before the glands were infected.

Micrococcus subgroups 3 and 6 were very common infections in teat canals throughout the herd and during all the lactation period. However, none caused intramammary infections and their distributions are not demonstrated.

The pathogenic significance of intramammary infections

The incidence of intramammary infections. Table 9 shows the types of bacteria isolated from milk taken by syringe through the teat wall and consequently of intramammary origin. Only one quarter remained completely uninfected throughout the lactation. Corynebacterium bovis was isolated from teat sinus milk in every other quarter in the herd at some time during the lactation, often concurrently with another bacterium. The incidence of S. aureus and coagulase negative coccal infections was low.

Three S. aureus and one Streptococcus uberis infections present at the time of calving caused clinical mastitis soon after calving and were treated. Two further clinical mastitis cases associated with S. aureus were treated in the third month of lactation and one in the fifth month. One cows' quarter contracted an intramammary infection of

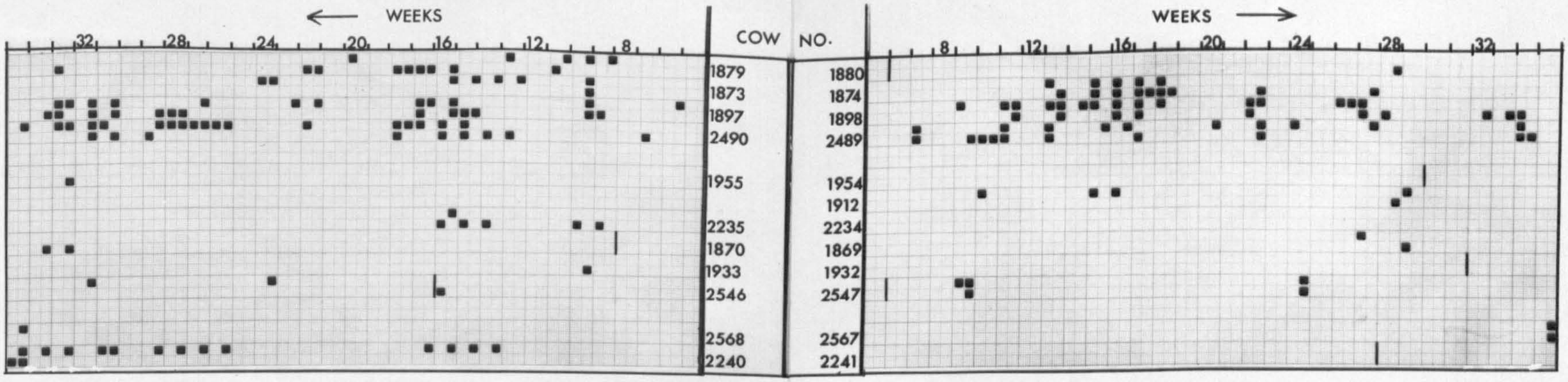


Fig. 40

Distribution of 1) S. subgroup 6.

TABLE 9 Types of intramammary infection

Type of bacteria present as intramammary infections	Period of lactations. Week numbers							
	1	2 - 8	1 - 12	13 - 16	17 - 20	21 - 24	25 - 28	29 - 32
	Number of quarters affected							
None	34	12	10	10	2	1	1	1
S.aureus	6	6	0	2	2	1	0	2
S.aureus and C.bovis	0	3	6	3	4	3	2	1
S.epidermidis	5	2	1	2	2	2	0	0
S.epidermidis and C. bovis	1	5	1	3	8	3	5	2
C.bovis	10	30	40	51	49	41	22	16
S.aureus and S.epidermidis	3	0	0	0	0	0	0	0
Total no. of quarters sampled	59	58	58	72	67	51	30	22
Number of quarters not sampled	13	14	14	0	1	17	2	2
No.of dry quarters	0	0	0	0	4	4	40	48
Total number of quarters	72	72	72	72	72	72	72	72

Streptococcus dysgalactiae near the end of lactation and this, although not causing clinical mastitis, was eliminated by treatment. There were no other intramammary streptococcal infections in the herd during the lactation and no other types of infection required treatment.

The changes in intramammary infections which occurred during lactation are shown in Table 10. It can be seen that the predominant bacterium was C. bovis which infected most quarters in the first half of the lactation, whether or not the quarter had an existing intramammary infection. Two quarters which were originally infected with S. epidermidis, became infected with C. bovis and the staphylococcus simultaneously disappeared. A similar event occurred in one quarter originally infected with S. aureus. In other quarters the corynebacterium established along with existing staphylococcal infections, and in some of these the staphylococci later disappeared.

In two quarters originally infected with C. bovis and later with S. aureus the corynebacteria subsequently disappeared. In only these and another two quarters in which C. bovis disappeared leaving the gland uninfected did C. bovis once present as an intramammary infection subsequently disappear. In all other quarters it persisted solely or in association with staphylococcal infections. Thus it appears that not only is C. bovis an extremely invasive organism but that once established within the udder parenchyma it is able to persist and is possibly associated with the elimination of existing infections.

TABLE 10 The changes in intramammary infections during lactation

Change in intramammary infection	Period of lactation: Week numbers						
	1 - 8	9 - 12	13 - 16	17 - 20	21 - 24	25 - 28	29 - 32
	Number of quarters						
Uninfected becoming infected with C. bovis	19	11	2	5	1	1	2
S.epidermidis becoming infected with C.bovis	0	0	0	2	0	0	0
S.epidermidis changed to C.bovis only	1	1	0	0	0	0	0
S.epidermidis C. bovis changing to C.bovis only	1	1	0	0	1	0	0
S.aureus becoming infected with C.bovis	3	2	3	0	0	1	0
C. bovis changing to S.aureus only	0	0	1	0	0	0	1
C. bovis becoming infected with S. epidermidis	0	0	1	0	0	0	0
C.bovis becoming uninfected	0	1	1	0	0	0	0
S. aureus and C. bovis changing to C. bovis only	0	1	0	0	0	0	0
S.aureus changing to C. bovis only	0	0	1	0	0	0	0

The pathogenicity of the intramammary infections as determined by cell counts

Table 11 records the geometric mean cell counts of uninfected quarters up to the time at which they first became infected and also quarters which were infected with C. bovis, S. epidermidis and with S. aureus. Because of the small numbers of quarters infected with S. aureus this group includes quarters which had a concurrent infection with C. bovis during all or part of the infected period.

Uninfected quarters As only one quarter remained uninfected throughout the lactation the number of values for cell counts at the end of the lactation relate to this one quarter.

The mean counts in this group rose steadily throughout the lactation. The rise which was apparent during the first eight weeks continued and became significantly higher by the intermediate period ($P<0.05$). The further rise after this period again became significant by the end of the lactation ($P<0.05$).

Quarters infected with C. bovis

The mean count for the intermediate period was significantly higher than those for the first 12 weeks ($P<0.01$). A further significant rise occurred by weeks minus 11 to minus 8 and also from weeks minus one to minus four to the last period of the lactation, ($P<0.01$).

Quarters infected with S. aureus

The cell counts in this group were high throughout the lactation and no significant changes occurred throughout the period. All the mean counts were above one million cells per ml.

TABLE 11

The changes in cell counts during lactation associated with different infections

Period of lactation	Mean cell counts per ml throughout lactation x 10^3							
Week numbers	Uninfected		C. bovis		S. aureus		S. epidermidis	
	Log mean ± S.E.M.	Geometric mean	Log mean ± S.E.M.	Geometric mean	Log mean ± S.E.M.	Geometric mean	Log mean ± S.E.M.	Geometric mean
1 to 4	2.347 ± 0.0716	222	2.755 ± 0.1026	578	3.067 ± 0.1801	1167	2.716 ± 0.0790	520
5 to 8	2.290 ± 0.0414	195	2.622 ± 0.0543	418	3.152 ± 0.717	1418	3.189 ± 0.1426	1546
9 to 12	2.422 ± 0.0638	264	2.612 ± 0.0284	409	3.211 ± 0.1047	1627	3.047 ± 0.149	1115
Inter-mediate	2.562 ± 0.0590	365	2.910 0.0181	813	3.176 ± 0.0566	1500	2.913 ± 0.0524	818
-11 to -8	2.602 ± 0.0819	400	3.022 ± 0.0220	1053	3.191 ± 0.0842	1553		
- 7 to -4	2.808 ± 0.1520	642	3.076 ± 0.0242	1191	3.187 ± 0.0788	1537	3.068 ± 0.0870	1170
- 3 to end	3.080 ± 0.1359	1203	3.171 ± 0.0264	1484	3.279 ± 0.0699	1899	3.1962 ± 0.1019	1451

Comparison of cell counts in relation to the type of infection present

The mean levels of cell counts in the quarters infected with C. bovis or S. aureus were both significantly higher than the level in uninfected quarters up to the weeks minus 11 to minus 8 ($P<0.01$). After this the other levels were higher but not significantly so. The mean cell count level from quarters infected with S. aureus was significantly higher than quarters infected with C. bovis up to and including the intermediate period. Thereafter the levels remained higher but were not significantly so.

S. epidermidis infections Because of the small numbers of quarters infected with S. epidermidis and the fact that some followed previous infections which had necessitated treatment, a full progression of cell counts throughout lactation is not possible. The available figures are shown in Table 11. The levels are similar to those from quarters infected with C. bovis.

Changes in cell count following intramammary invasion with C. bovis

An analysis of weekly cell counts of uninfected quarters showed a marked rise near the time at which C. bovis was first isolated from teat sinus milk. A comparison was made between the cell counts before and after invasion of the gland and the results showing the changes in relation to the period of the lactation at which it occurred are shown in Table 12. In each case there was a highly significant mean increase shortly after infection.

Table 12

The changes in mean cell counts associated with *C. bovis* infection in mammary glands

Weeks preceding infection			Weeks following infection		
Week No. of lactation	Cell count x10^3		Week No. of lactation	Cell count x 10^3	
	Log mean	Geometric mean		Log mean	Geometric mean
1 - 4	2.290	195	5 - 8	2.658	455
9	2.39	247	10 - 12	2.760	575
17 - 18	2.68	483	19 - 20	3.40	2540

Discussion

The main fact which emerges from this study of intramammary infections is the invasiveness of C. bovis. It is then associated with mean cell counts higher than the mean counts from uninfected quarters. This work was done on the same herd as that used by Forbes and Hebert 1968, in which staphylococci were the main intramammary bacteria, the only difference being that 4 cows had been culled. C. bovis was introduced inadvertently into the herd between lactations by another cow which was also culled before the start of the second lactation. Despite reasonable hygienic precautions during milking the organism quickly spread throughout the herd. The marked difference in the incidence of staphylococcal infections in the two lactations is probably associated with the introduction of C. bovis. This could not be proved experimentally as possible control animals quickly acquired the corynebacterial infection but the incidence of staphylococcal teat canal infections throughout the lactation period described here was high. Presumably the staphylococci invaded the gland with the usual frequency but could not establish as intramammary infections due to the presence of C. bovis. Newbould and Neave (1965a), showed that S. aureus when inoculated into glands infected with corynebacteria was not recovered 10 minutes later with the frequency that it was from uninfected glands. They attributed this to a higher number of leucocytes in the milk rather than to the presence of the corynebacterium itself. In the work reported here the mean cell levels in quarters infected with C. bovis were higher

than in uninfected quarters and it is probable that the leucocytes were responsible for the failure of staphylococcal organisms to establish in the udder parenchyma. In addition the disappearance of existing staphylococcal infections was sometimes associated with a new infection of corynebacteria and it is possible that the inflammatory response of the gland alters in some way to cause expulsion of staphylococci. It is of interest that those types of S. epidermidis infections which did occur all provoked an obviously high cell count and there was not any infection of a non-pathogenic type described earlier (Forbes ~~et al~~ and Hebert 1968). This is probably due to such types having a greater susceptibility to leucocytes than pathogenic types.

The only bacteria to cause clinical mastitis in this work were recognised pathogens i.e. S. aureus and Str. uberis, but most quarters had intramammary infections with other bacteria. This would seem to confirm that the facility with which bacteria invade the mammary gland is unrelated to their pathogenicity within it.

The passage of bacteria through the teat canal is not fully understood, but it is probably dependent upon the weight and type of infection on the teat skin and in the teat canal. The subsequent establishment of infection within the gland is further dependent upon the inherent pathogenicity of the organism and the susceptibility of the host. This in turn may be influenced by the numbers of leucocytes in the milk as a result of an existing or recent intramammary infection. In investigating the mechanisms by which pathogens colonise the udder parenchyma the role of "commensal" bacteria cannot be ignored.

Part IX

The passage of staphylococci through the bovine teat canal

Summary

In order to determine how bacteria pass through the teat canal into the teat sinus, milk samples were taken by syringe collection through the teat wall from 12 quarters of seven cows before and after each milking for up to 16 days. Three of the teat canals were naturally infected before the start of the experiment and the remainder were artificially infected with S. aureus by the Hadley-Wisconsin swab technique. In three of the quarters the inoculum was introduced into the teat sinus through the teat canal by the inoculation technique although the swabs were inserted only 3-5 mm. into the canal. The other 9 quarters of the 12 were milked a total of 149 times during the experiment but only once was a colony-forming unit of the bacterium present in a teat canal infection isolated from the milk taken from the teat sinus after milking. One quarter developed an intramammary infection, the bacterium first being detected in the teat sinus prior to the third milking after inoculation of the teat canal. The invasion of the bacterium into the teat sinus therefore occurred between milkings. S. aureus persisted in teat canals between 5 and 32 or more milkings after inoculation. The experiment showed that bacteria in the teat canal are seldom refluxed into the residual milk in the teat sinus during correctly conducted milking with an efficient machine.

Introduction The mechanism by which bacteria invade the mammary gland is little understood. It is suspected that invasion occurs by bacteria growing through the teat canal or that bacteria are refluxed through the canal into the teat sinus during milking. Murphy and Stuart (1953b) working with Streptococcus agalactiae, showed that the teat canal could remain infected for up to 9 days before an intramammary infection developed. In the period during which the infection was confined to the teat canal the authors suspected significant bacterial growth to the end that they grew through into the teat cavity, but stressed that more information was needed before such a conclusion could be justified. Newbould and Neave (1965a) who inoculated teat canals with Staphylococcus aureus found that when colonisation of the teat canal occurred, only rarely did intramammary infections follow. The method by which the bacterium invaded the gland was not established. The evidence for reflux of bacteria through the teat canal in normal conditions is equivocal. Little (1937) thought that bacteria were sucked through the teat canal in his experiments but his methods could not verify this. McEwan and Samuel (1946) demonstrated that bacteria passed through the teat canal into the teat sinus when a broth culture was sprayed onto the teat ends during milking. The necessary physical conditions for reflux to occur were produced by Witzel and McDonald (1965) who, by continuing milking after complete milk removal recorded a residual vacuum in the teat sinus after removal of the teat cups. They suggested that bacteria may enter during pressure equilibration. In a herd in which normal machine milking

was practised Forbes and Hebert (1968) showed that teat canals were infected for some time before the development of most intramammary infections although others developed without detectable preceding teat canal infections. The results suggested that infection grew through the teat canal in most cases but reflux of bacteria may have occurred in others. In subsequent work done on the herd as reported in Part VII of this thesis, it was suspected that the intramammary environment may have been sufficiently changed by the presence of C. bovis to prevent establishment of Micrococcaceae infections. Therefore, in order to detect when bacteria penetrated into the gland it was necessary to demonstrate their presence at the time of penetration if possible. The experiment reported here was designed to determine how bacteria in the teat canal invade the teat sinus.

Materials and methods

Cows Seven animals were used in the experiment. Four were heifers, two in their third, and one in her fifth lactation. Twelve quarters were used for teat canal inoculation study, all of which had intramammary infections of Corynebacterium bovis at the start of the experiment. There was no erosion of the teat canals. During the experiment each animal was milked by a sterile machine working at 13 in. Hg vacuum operated at 60 cycles per min and with a pulsation ratio of 1:1. The interval between morning and evening milkings was 8 hours.

Teat canal inoculation

Test bacterium An α haemolytic Staphylococcus aureus having a phage pattern of 29, 110+ (Davidson 1961) was used. It was isolated from a cow with mastitis. Its phage type and haemolytic pattern distinguished it from other strains of S. aureus present in the experimental herd.

Inoculation procedure Preliminary work showed that the Hadley Wisconsin swab technique (Murphy and Stuart 1953b) was a satisfactory method of introducing a large inoculum into the teat canal. Each teat apex was thoroughly cleaned with sterile cotton wool soaked in methylated spirits. A swab was then loaded with S. aureus by drawing it through several colonies of a 24 hour culture on 5% sheep blood agar and a teat canal was inoculated by inserting the swab between 3 and 5 mm. into the canal.

Uninoculated quarters At the start of the experiment three quarters had teat canals naturally infected with Micrococcaceae. The right fore quarter of cow 2240 was infected with a Staphylococcus subgroup 6., the right fore of cow 2816 with a Staphylococcus subgroup 3 and the right

fore of cow 2490 with a mixed infection of Micrococcus subgroups 4 and 5 determined by the classification of Baird-Parker (1963).

Sampling procedure Immediately before every milking the teat skins and teat apices were thoroughly cleaned with sterile cotton wool soaked in methylated spirits. Before afternoon milking a sterile swab was inserted 3 mm into the teat canal, inoculated into 5 ml of 1 per cent glucose broth by stirring and then discarded. This was done before the collection of any other samples. Milk samples were taken after similar preparation of the teats immediately before and after every milking. One ml of milk was collected by syringe from the teat sinus using the method of Murphy and Stuart, (1954c). This was followed by the normal collection of approximately 10 ml of foremilk drawn through the teat canal.

Cultural technique 0.1 ml samples were plated within 30 min. of collection, onto 5% sheep blood agar plates. Teat canal swab broths and hand-drawn milk samples were inoculated on to separate plates. Five plates were seeded each with 0.1 ml of the milk collected by syringe through the teat wall. All plates were incubated at 30°C for 48 hours and refrigerated before being read. One colony of the test bacterium from each series of samples (e.g. pre-milking samples) was identified by the tube coagulase test and phage typing in the case of S. aureus, or by biochemical methods for coagulase negative types (Baird-Parker, 1963).

Results

The nine quarters whose teat canals were artificially inoculated were sampled between three and seven milkings before inoculation. The milk samples taken by teat wall puncture during the period preceding inoculation produced only C. bovis when cultured. Of the nine teat canals inoculated with S. aureus, in three the organism was inadvertently inserted through the canal into the teat sinus. This was shown by the fact that in each of these quarters the milk sample taken by teat wall puncture after inoculation contained the test organism. Two of the quarters had the teat wall puncture samples taken immediately after inoculation but in the third the right fore of cow 2240 the interval was 16 hours between evening and morning milkings, during which time the bacterium may have penetrated into the teat sinus. Table 13 shows the details of the nine quarters which were inoculated. In the six inoculated quarters that were not followed by immediate or rapid penetration of the teat sinus, five quarters were sampled until it was evident that the test bacterium had disappeared. The sixth quarter, the left fore of cow 2939 in whose teat canal S. aureus persisted was sampled for sixteen days following inoculated and the experiment was then terminated. Two quarters with uninoculation teat canals were sampled for eight days and one for 16 days.

S. aureus persisted for between 5 and 32 or more milkings following inoculation (Table 13). In those teat canals where S. aureus persisted for 5 milkings, there was a rapid decline in colony counts in hand-drawn milk and from teat canal swabs. In the other quarters counts showed an increase in numbers of bacteria and a subsequent decline and eventual

Table 13

Inoculation of S. aureus into the lower 3-5mm of teat canals

Cow	Quarter	Time of teat canal inoculation	Result of Inoculation
2816	LF	Before pre p.m. milking samples	
2706	RF	Before pre p.m. milking samples	Bacterium introduced into the teat sinus by inoculation
2240	RH	After post p.m. milking samples	
2940	RF		Bacterium in teat canal for 17 milkings
2939	LF		Bacterium in teat canal for 32 milkings
2939	RH	Before post p.m. milking samples	Bacterium in teat canal for 11 milkings
1880	LF		Bacterium in teat canal for 5 milkings
1880	RF		Intramammary infection at 3rd post inoculation milking
2567	RF	After post p.m. milking samples	Bacterium persisted in teat canal for 5 milkings

disappearance. The left fore teat canal of cow number 2939 which was still infected after 16 days was tested a further 14 days later but the test bacterium had by then disappeared.

Coagulase negative strains The mean number of colony forming units on plates prepared from broths inoculated with teat canal swabs from the three quarters with naturally occurring teat canal infections was 31.5. This indicated the collection of approximately 630 colony forming units per swab throughout the period of sampling.

Passage of Bacteria through the Teat Canal in Inoculated Quarters

1) Reflux of bacteria during milking

Six quarters with S. aureus infections in the teat canal were milked a total of 85 times during the course of the infection. In paired milk samples collected by teat wall puncture before and after each milking, only once was the bacterium present in the teat sinus milk. . One colony was present on one of the five plates prepared from the sample taken from the left fore teat sinus of cow number 2940 after the third milking following inoculation of the canal. The hand-drawn milk samples before and after this milking had colony counts of 300 and 2 respectively in 0.1 ml. During the 14 subsequent milkings of this quarter when S. aureus was in hand-drawn milk, the bacterium was not again isolated from the teat sinus. Apart from this incident there was no evidence of reflux of bacteria having occurred during milking.

2) Passage of bacteria between milkings

The right fore quarter of cow 1880 developed an intramammary infection after teat canal inoculation, and the progress of the infection is shown in Table14. In the two days following inoculation there was a progressive decline in colony counts of S. aureus from both normally drawn milk and teat canal swabs. Before the third milking after inoculation the test bacterium was isolated from milk taken by teat wall puncture, and the infection was again present in the teat sinus before the fifth milking. Invasion of the teat sinus had therefore occurred between milkings. After the fifth milking an intramammary infection became established but the bacterium was no longer recovered from swabs inserted into that part of the teat canal where the inoculum had been deposited. Thus, although the bacterium passed through the teat canal there was little evidence of colonisation in the canal before invasion of the teat sinus milk.

Traversal of Bacteria across the Teat Canal in uninoculated quarters

The strain of bacterium isolated from teat canal swabs and milks of the three uninoculated quarters was not isolated from any teat wall puncture samples during the experiment. The three quarters were tested for a total of 64 milkings.

Table 14

The development of intramammary infection after inoculation of the teat canal with S. aureus

Time of sampling	Cow No.1880 RF. S.aureus colony count of milk collected before and after milking		S. aureus colony counts from Teat canal swab /
	Teat wall puncture milk*	Hand drawn milk*	
Day 1 Pre a.m.	0	0	-
Post a.m.	0	0	-
Pre p.m.	0	0	0
Post p.m.	0	No. sample ≀	-
Day 2 Pre a.m.	0	7500	-
Post a.m.	0	250	-
Pre p.m.	0	90	10,000
Post p.m.	0	0	-
Day 3 Pre a.m.	8	400	-
Post a.m.	0	0	-
Pre p.m.	0	30	300
Post p.m.	0	0	-
Day 4 Pre a.m.	30	0	-
Post a.m.	250	10	-
Pre p.m.	80	30	0
Post p.m.	0	10	-
Day 5 Pre a.m.	400	150	-
Post a.m.	200	30	-
Pre p.m.	500	500	0
Post p.m.	60	0	-
Day 6 Pre a.m.	100	1000	-
Post a.m.	500	100	-
Pre p.m.	500	750	0
Post p.m.	50	200	-
Day 7 Pre a.m.	250	150	-
Post a.m.	300	1000	-
Pre p.m.	150	250	0
Post p.m.	40	20	-
Day 8 Pre a.m.	50	90	-
Post a.m.	500	100	-
Pre p.m.	1000	1000	0
Post p.m.	-	-	-

*Estimated count per ml. of milk
/Number of colonies per swab
≀Teat canal inoculated before post p.m. samples

Discussion

The Hadley Wisconsin swab technique was chosen as the method of inoculating the teat canal because it allowed a large inoculum to be used and also avoided introducing fluid into the teat canal. Bartley+Allen Slanetz (1965) thought it improbable that the introduction of small volumes of broth into the teat canal caused penetration into the teat simus. However, as the inflammatory response was delayed for only up to one day by ~~this~~ their method of inoculation it did not simulate the natural teat canal infections which preceded intramammary infection reported by Forbes and Hebert (1968). Newbould and Neave (1965a) also introduced a fluid inoculum 5 mm into the teat canals and using between 70 and 600 colony forming units of S. aureus, were able to recover the bacterium for up to 6 milkings or more. In those quarters which developed intramammary infections the delay in elevation of cell count was up to 6 milkings following inoculation. In another paper (Newbould and Neave 1965b) describing experiments in which the same strain of S. aureus was introduced directly into the teat sinus, the leucocyte response was in one case delayed up to the eleventh milking and was never evident before the third post inoculation milking. Hence, it is possible that in those quarters in which S. aureus was introduced into the teat canal and which developed intramammary infection the inoculum may have penetrated into the teat sinus at the time of inoculation. In the work reported here a swab inserted 5 mm into the teat canal was sufficient to introduce infection into the teat sinus in two quarters.

There is always a delay between bacterial invasion of the mammary gland and the inflammatory response as shown by an elevation of cell numbers in the milk. Therefore, in order to detect the time of bacterial invasion of the gland, methods which demonstrate the bacteria to be present within the gland must be used. McEwan and Samuel (1946) showed (by post mortem examination) bacteria to have entered the gland ~~by post mortem examination~~ immediately after milking. During milking the teat ends had been heavily contaminated with a motile bacterium which may have assisted entry into the gland. The method of collecting milk by syringe through the wall of the teat is the only way of detecting bacteria in the gland of the living animal. By comparing the bacteriology of samples taken immediately before and after milking the time of entry of bacteria can be determined.

In the work reported here, milk samples were plated within 30 min. of collection during which time phagocytosis of bacteria may have occurred. However, Katsube and Blobel (1964) showed that S. aureus, although phagocytosed by leucocytes, survived and were able to grow when the bacteria and leucocyte suspension was plated on sheep blood agar. Newbould and Neave (1965c) on the other hand showed that growth could be prevented if the bacterium was ingested by leucocytes suspended in milk when incubated for 18 hours. Only when the leucocytes were disrupted did bacterial multiplication continue. Culturing on solid media will not maintain viable leucocytes and ingested bacteria survive and form normal colonies. The limitations of the cultural methods used here may be in their insensitivity as only 0.5 ml. of milk was cultured and if very small numbers of bacteria penetrated into the sinus they may have escaped detection.

Little (1937) suspected that where invasion occurred, bacteria had been sucked through the teat canal. He recognised invasion by the development of mastitis but his methods could not establish that bacteria had been sucked into the teat sinus. The work of Witzel and McDonald (1964) who reported a residual vacuum in the teat sinus after complete milk removal by machine producing conditions in which reflux of bacteria could occur, was queried by Theil, Clough and Dodd (1965) who suggested that the vacuum was an artefact due to the cluster weight. However, to produce such a vacuum at all would demand extreme overmilking and during the work reported here the cows were milked normally and were not stripped out. The milk yields of the cows did not drop during the experiment showing that normal milking was practised. In addition the machines were operated with an adequate reserve and stable vacuum. Wilson (1958) deduced that a fluctuating reserve vacuum may predispose to mastitis and Nyhan and Cowhig (1967) showed that a low vacuum reserve was associated with a high prevalence of mastitis. They thought the fluctuation in vacuum at the end of the teat and consequent slowing of milking rate may provide the physical conditions necessary for bacteria to gain entry to the teat cistern from the teat canal or exterior of the teat. The experiment reported here showed that bacteria in the teat canal are seldom refluxed into the residual milk in the teat sinus by correctly conducted milking with an efficient machine. During only one of 149 milkings was there evidence of bacteria reaching the teat sinus during milking.

The right forequarter of 1880 developed an intramammary infection and the bacterium was first detected in the teat sinus in the sample taken prior to the third post inoculation milking (Table 14). Based on colony counts in normal milks after inoculation, there was little multiplication of the bacterium within the teat canal. On the third day after inoculation S. aureus was not recovered by swabbing that part of the teat canal in which the inoculum was deposited although the gland itself was then infected. The bacterium was isolated from the teat sinus before the third and fifth milkings after inoculation, after which all teat wall puncture samples were infected. Invasion of the teat sinus therefore probably occurred between milkings but due to the inability of the bacterium to colonise the teat canal it is doubtful if growth occurred through the canal. It is possible that the infection was carried through the canal in the keratin lining as a consequence of pressures imposed on the teat during milking. Thiel, Clough, Westgarth and Akam (1966) showed that at certain pulsation rates the teat liner closes the teat sphincter in the rest phase of milking. This pressure may cause keratin to move in the canal. Eversion of keratin occurs giving eroded teat orifices in some quarters and it may be that the structure of some teat canals and conformation of some teats are such that inversion of keratin occurs. If the keratin is infected the process would then effect the passage of bacteria into the sinus. Pounden and Grossman (1950) found that machine milking removed more keratin from the teat canal than did hand milking or calf suckling, especially if overmilking was practised.

A predisposition to teat erosion in pointed teats and in teats with

plate formed tips was found by Johansson (1957), while Hickman (1964) found a striking trend of increased incidence of mastitis with increase in teat diameter. He also found that funnel shaped teats had a lower frequency of mastitis than had cylindrical shaped teats. It may be that mechanical stresses imposed on some teats by machine milking may be a factor in introducing infection into the gland from the teat canal.

General Discussions and Conclusions

The purpose underlying this thesis was to investigate the mechanism by which bacteria passed through the teat canal and initiated infections in mammary parenchyma. Preliminary observations had shown that before intramammary infections developed the bacteria often had been resident in the teat canals for periods up to several weeks duration. Consequently teat canal infections were investigated in some detail. It was known that Micrococcaceae were common inhabitants of teat skin and the teat canal. S. aureus can be easily identified by its coagulase reaction and can also be accurately phage typed by which separate strains can be recognised. Coagulase negative strains cannot be so minutely defined but a recent classification (Baird-Parker 1963) did enable types to be identified well enough for their epidemiology to be investigated.

The study was rewarding for it showed that a ubiquitous population of coagulase negative Micrococceae was in fact composed of bacterial subgroups which fluctuated in importance throughout the sampling period. The distribution of infections within the herd was related to the standing positions of the cows in the byre. This showed that when a bacterial subgroup became ascendant it was often isolated first from one or two particular animals and subsequently spread in a manner suggesting that the nearest animals were most often infected first. This could not be established statistically but the patterns were repeated regularly enough for this to be probable. The only skin site investigated in this work was the teat canal so it has not established the progression by which

bacteria first establish in the teat canal. It is probable that any skin site would have shown similar trends.

Adjacent skin sites probably become infected by physical contact whether of the same or another animal. Close proximity without actual contact may be sufficient, especially if the cattle are groomed with a consequent dispersal into the atmosphere of epithelial debris. The transfer of bacteria to the teat canal may also be effected by washing the udder since Beech (1966) showed this practice to increase the numbers of bacteria at the teat end. He suggested that the washing fluid mobilised infection from the udder which then gravitated to the teat end carrying bacteria with it. ~~However~~ By whatever means bacteria invade teat canal keratin it is a very suitable environment for those bacteria which inhabit skin. Keratin collected along with its natural bacterial population was incubated in vitro in conditions simulating the teat canal environment. Usually the bacteria continued to survive for a month or more. As most bacterial subgroups were isolated from the material it is doubtful if whole keratin is bactericidal. However, it is possible that some keratin samples were bactericidal to bacterial types other than those which were isolated from them. A point of interest during this work was the observation that bacteria in keratin were probably unable to permeate through the material by growth. This was concluded from the appearance of bacteria in sub-cultures taken from the material at intervals up to 27 days after collection when they had not previously been detected. There was also a parallelism of infections in keratin and the teat canals from which it was harvested

when sampled at similar times. This was particularly evident in the case of S. subgroup 6 infections. The physical nature of keratin makes it extremely doubtful that bacteria, particularly non-motile bacteria could move significantly within it without mechanical assistance. This may be important when considering how bacteria pass through the teat canal which has a mean length of approximately 11 mm. It has been suggested that bacteria grow through it which suggests a progressing bacterial front up the canal. The work in this thesis has shown that bacteria certainly grow within the canal but it is doubtful if they grow through it; at least without mechanical assistance. The author concluded after reviewing literature on the passage of bacteria through the teat canal, that keratin may be moved up the canal by the mechanics of machine milking, particularly in certain shaped teats. It has also been shown that keratin maintains viable bacteria for prolonged periods. Therefore it is possible that this is the mechanism by which bacteria traverse the canal. It is consistent with previous work (which was again confirmed) that teat canals are often infected for weeks before intramammary infections develop since it is probable that the intrusion of keratin from the external meatus of the teat canal to its proximal end is a slow process. It is also consistent with the fact that healthy cows, milked under hygienic conditions by properly adjusted and maintained machines do nevertheless develop mastitis. It is supported by other results which showed no evidence of bacteria being sucked into the teat sinus by properly conducted machine milking. This does not preclude the

possibility that overmilking or using machines with a fluctuating vacuum may enable bacteria to be sucked through the teat canal. However, two recent publications suggest this is unlikely to occur.

McDonald and Witzel (1968) showed that after milk flow ceased, there was no fluctuation of vacuum at the teat end while Cowhig, Nyham, Phillips and O'Flaherty (1968) concluded that any residual vacuum in the teat sinus after overmilking is unlikely to be relieved by aspiration through the teat canal, but more likely by the passage of milk from the gland sinus. This suggests that even such malpractice as overmilking may not facilitate the passage of bacteria into the gland.

However, it should be remembered that overmilking does cause excess removal of keratin (Pounden ~~et al~~ and Grossman 1950 , Walser 1966) and if it is intruded in some teats this could effect bacterial invasion of the gland. This may be especially important during overmilking because once milk flow ceases the teat canal does not open, and there is no vehicle to carry away any eroded keratin. It is hoped to continue this work by developing teat cups which do not allow marked fluctuations of vacuum at the teat end or complete collapse of the liners. Controlled experiments could then establish if there was any difference in the rate of new infections between cows milked with these teat cups and conventional models.

A difficulty which was experienced in this work in common with a lot of other work in mastitis, was the recognition of intramammary infections. Precise methods to detect bacteria in the gland at the earliest possible moment after infection as used in Part IX of this thesis cannot be

continued for long. It is therefore necessary to recognise mastitis, using the most sensitive laboratory tests available and then interpreting the results to suggest a probable time of bacterial invasion. However, these methods are still incredibly vague, especially when attempting to investigate a pathogenetic process which may be of several weeks duration. Neither can it be ascertained if signs of mastitis follow the first bacterial penetration into the gland. Various workers have shown that inoculation of bacteria into fully susceptible udders does induce mastitis generally of an acute type. Schalm's work has shown that the level of cells in milk markedly alter the susceptibility of the gland to infection and the work in Part VIII of this thesis showed that bacteria which very seldom cause clinical mastitis are quite pathogenic when accurate measurements of inflammation are made. This was particularly the case with C. bovis whose incidence was high in the experimental cows.. It is fairly certain that such bacteria do increase the resistance of the gland to invasion by more pathogenic types. This was not proved because C. bovis spread so quickly that the rate of new infections with other bacteria could not be assessed in animals infected and uninfected with the bacterium. However, the proportion of glands with teat canals infected with Micrococcaceae which developed intramammary infections was lower than was expected from previous results when C. bovis was not endemic in the herd. (Forbes and Hebert 1968). It is probable that invasion of the gland occurred but infection did not establish due to large numbers of cells present consequent to C. bovis infection eliminating the invading

organisms by phagocytosis.

In investigating the manner in which bacteria invade the mammary gland it is important not only to observe pathogenic bacteria but also those others which may influence the host's susceptibility. Ideally udders which are sterile and have no history of previous infection should be used for studies on the pathogenesis of mastitis. C. bovis being apparently so invasive may be a good species for examining the penetration through the teat canal. However, it may not be any more invasive except by weight of infection than other common intramammary inhabitants since part of this work showed that it may remain localised in the teat canal for more than a month before being isolated by syringe from the teat simus. It is likely that however bacteria invade the gland, the mechanism is the same for all non-motile bacteria. The difficulties of investigating this have been shown and it may still be necessary to test theories of invasion using the incidence of mastitis as the parameter rather than showing unequivocally the passage of bacteria into the gland.

REFERENCES

ABD-EL-MALEK, Y. and GIBSON, T. (1952). Studies in the bacteriology of milk III. The Corynebacteria of milk. J. Dairy Res. 19 153.

ADAMS, E.W. and Rickard, C.G. (1963). The anti-streptococcic activity of bovine teat canal keratin. Am. J. vet. Res. 24 122.

ADAMS, E.W., RICKARD, C.G. and MURPHY, J.M. (1961). Some histological and histochemical observations on bovine teat epithelium. Cornell Vet. 51 124.

ANDREAE, U. (1958). Messungen am Zitzenkanal von Kühen zur Ermittlung der Melkbarkeit. Z. Tierzucht. ZüchtBiol. 71 289.

ARDRAN, G.M., KEMP, F.H., CLOUGH, P.A. and DODD, F.H. (1958). Cineradiographic observations on machine milking. J. Dairy Res. 25 154.

ARNOLD, J.P. (1950). Anatomy and pathology of the bovine teat. J. Am. vet. med. Ass. 116 112.

AUCLAIR, J.E. and HIRSCH, A. (1953). The inhibition of microorganisms by raw milk. 1 The occurrence of inhibitory and stimulatory phenomena. Methods of eestimation. J. Dairy Res. 20 45.

BAIRD-PARKER, A.C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. J. gen. Microbiol. 30 409.

BAIRD-PARKER, A.C. (1965a). The classification of staphylococci and micrococci from world wide sources. J. gen. Microbiol. 38 363.

BAIRD-PARKER, A.C. (1965b). Methods for classifying staphylococci and micrococci. In "Identification methods for Microbiologists." Ed. Gibbs, B.M. and Skinner, F.A. Academic Press, London.

BANG, B. (1888) - cited by Christiansen and Neilson, 1934.

BARRITT, M.M. (1936). The intensification of the Voges-Proskauer reaction by the addition of alpha-naphthol. J. Path. Bact. 42 441.

BAUMGARTNER, H., KASTLI, P., WALSER, V. and MEEDER, K. (1965). Versuche über die Bakteriyidie der Milch. Pathalogia Microbiol. 28 3.

BAXTER, E.S., CLARKE, P.M., DODD, F.H. and FOOT, A.S. (1950). Factors affecting the rate of machine milking. J. Dairy Res. 17 117.

BEECH, J.A. (1966). Personal communication.

BEECH, J.A. (1967). A study of the results obtained from bacteriological and cytological examination of various portions of cows milk. Vet. Rec. 80 667.

BEECH, J.A. and FORBES, D. (1965). Pathogens in the streak canal. Vet. Rec. 77 1085.

BENDIXEN, H.C. (1932). On the corynebacteria occurring in milk from clinically normal cow udders. Acta path. microbiol. scand. Suppl.11. 187.

BERGER, J. and FRANCIS, J. (1951). Mastitis in practice. 1. Epizootiology and bacteriology. Vet. Rec. 63 283.

BERNHEIMER, A.W. (1965). Staphylococcal alpha-toxin. Ann. N.Y. Acad. Sci. 128 112.

BLACKBURN, P.S. (1966). The variation of cell count of cow's milk throughout lactation and from one lactation to the next. J. Dairy Res. 33 193.

BLANK, I.H. and DAWES, R.K. (1958). The water content of the stratum corneum IV. The importance of water in promoting bacterial multiplication on cornified epithelium. J. invest. Derm. 31 141.

BLOBEL, H. and KATSUBE, Y. (1964). Effects of experimentally induced leucocytosis in bovine mammary glands upon infections with S. aureus Str. agalactiae and A. aerogenes. Am. J. vet. Res. 25 1085.

BOGE, A. (1965). Untersuchungen über verschiedene pradisponierende Faktoren für die Enstchung von Mastitiden. Inaug. Diss. Hanover.

BOULAND, C.T., MARSHALL, R.T., HINDERY, G.A. and TURNER, C.W. (1967). Mastitis due to C. bovis and oestrogen interaction. J. Dairy Sci. 50 978.

BRATLIE, O. (1966). Environment and bovine mastitis. Nord. VetMed. 18 366-375.

BROWN, R.W., SANDVIK, O., SCHERER, R.K. and ROSE, D.L. (1967). Differentiation of strains of S. epidermidis isolated from bovine udders. J. gen. Microbiol. 47 273.

BULL, L.B., MUNCH-PETERSON, E., MURNAME, D. and McLEAN, J.D. (1940). Bull. Coun. Sci. indust. Res. Aust. No.134.

BURTENSHAW, J.M.L. (1938). The mortality of the haemolytic streptococcus on the skin and on other surfaces. J. Hyg., Camb. 38 575.

BURTENSHAW, J.M.L. (1942). The mechanism of self-disinfection of the human skin and its appendages. J. Hyg., Camb. 42 184.

CAMPBELL, B., PORTER, R.M. and PETERSON, W.E. (1950). Plasmacytosis in the bovine udder during colostrum secretion and experimental cessation of milking. Nature, Lond. 166 913.

CARROLL, E.J., SCHALM, O.W. and LASMANIS, J. (1965). The use of a synthetic cortiooid on experimental coliform mastitis in cattle. The effects of intramammary and intramuscular administration on the inflammatory response. Am. J. vet. Res. 26 858.

CARUOLO, E.V., and MARX, G.D. (1962). Comparison of the vacuum applied to the teat cup and that measured within the gland and teat cisterns of the lactating bovine mammary gland. J. Dairy Sci. 45 696.

CHRIST, F. (1905). Research on the musculature and the elastic connective tissue in the mammary gland of domestic animals. (Title trans) Cited by Turner 1939.

CHRISTIANSEN, W. (1929). Milchw. Forsch. 7 233 oited by Munch-Petersen. E. Bovine Mastitis. Imp. Bur. An. Hlth Weybridge.

CHRISTIANSEN, M. and NIELSEN, F. (1934). Infectious bovine mastitis. Proo. 12th Int. Vet. Congr. 2 548.

COBB, R.W. and WALLEY, J.K. (1962). Corynebacterium bovis as a probable cause of bovine mastitis. Vet. Rec. 74 101.

CORNBLEET, T. (1932). Self-sterilising of the skin II. The body folds. Archs Derm. Syph. 25 1058.

COWAN, S.T. (1962). An introduction to chaos, or the classification of micrococcoi and staphylococcoi. J. appl. Bact. 25 324.

COWAN, S.T. and STEEL, K.J. (1965). Manual for the identification of medical bacteria. Cambridge University Press.

COWHIG, M.J., NYLAN, J.F., PHILLIPS, D.S.M. and O'FLAHERTY, T. (1968). Pressure changes in teat sinus at end of machine milking. J. Dairy Sci. 51 912.

CULLEN, G.A. (1966). The ecology of Str. uberis. Br. vet. J. 122 333.

CULLEN, G.A. (1966). Cells in milk. Vet. Bull., Weybridge. 36 337.

CULLEN, G.A. (1967a). Short term variations in the cell count of cows' milk. Vet. Rec. 80 649.

CULLEN, G.A. (1967b). A method of counting cells in milk using an electronic cell counter. Vet. Rec. 80 188.

CULLEN, G.A. and HEBERT, C.N. (1967). Some ecological observations on micro-organisms inhabiting bovine skin, teat canals and milk. Br. vet. J. 123 14.

DAVIDSON, I. (1961a). Observations on the pathogenic staphylococci in a dairy herd during a period of six years. Res. vet. Sci. 2 22.

DAVIDSON, I. (1961b). A set of bacteriophages for typing bovine staphylococci. Res. vet. Sci. 2 396.

DAVIDSON, I. (1967). Personal communication.

DAVIDSON, I. and SLAVIN, G. (1958). The disinfection of milking units in the control of mastitis due to Str. agalactiae. Vet. Rec. 70 893.

DAVIDSON, I., SLAVIN, G. and STUART, P. (1954). An experimental study of the control of Str. agalactiae infection in dairy cattle. Vet. Rec. 66 466.

DAVIS, D.J. (1935). The dissemination of human pathogenic streptococci through the cow's udder. J. Bact. 29 42.

DARBYSHIRE, J.B. (1960). Experimental studies on the occurrence of staphylococcal antitoxin in the whey of cows. Res. vet. Sci. 1 350.

DARBYSHIRE, J.B. (1962). Immunity in bovine mastitis. Vet. Bull., Weybridge. 32 1.

DARBYSHIRE, J.B. (1964). The multiplication of S. aureus in the milk of cows with mild mastitis. J. Path. Bact. 87 137.

DILLON, W.M., BROWN, C.M., ALBRIGHT, J.L., CALLAHAN, C.J. and WEINLAND, K.M. (1967). Influence of wide and narrow milking machine inflations on / udder health. J. Dairy Sci. 50 992.

DODD, F.H. and NEAVE, F.K. (1951). Machine milking rate and mastitis. J. Dairy Res. 18 240.

DODD, F.H., OLIVER, J. and NEAVE, F.K. (1957). The effect of design of teat cup liners on mastitis incidence. J. Dairy Res. 24 20.

DUTHIE, E.S. (1954). Evidence for two forms of staphylococcal coagulase. J. gen. Microbiol. 10 427.

EDWARDS, S.J. and JONES, G.W. (1966). The distribution of characters of coagulase-negative staphylococci of the bovine udder. J. Dairy Res. 33 261.

EDWARDS, S.J. and SMITH, G.S. (1959). Staphylococcal antitoxin in the blood and milk of cattle. J. comp. Path. 69 203.

ELEK, S.D. and LEVY, E. (1950). Distribution of haemolysins in pathogenic and non pathogenic staphylococci. J. Path. Bact. 62 541.

ELLIOTT, L.P. (1965). Staphylococci of bovine mastitis: their ecology in the dairy herd and its environment. Characterisation of the isolates. Ph.D. Thesis. Wisconsin.

ESPE, D. and CANNON, C.Y. (1942). The anatomy and physiology of the teat sphincter. J. Dairy Sci. 25 155.

EVANS, A.C. (1915). Bacillus abortus in market milk. J. Wash. Acad. Sci. 5 122.

EVANS, A.C. (1916). The bacteria of milk freshly drawn from normal udders. J. infect. Dis. 18 437.

EVANS, A.C. (1918). Further studies on Bacterium abortus and related bacteria III. B. abortus and related bacteria in cow's milk. J. infect. Dis. 23 354.

EVANS, J.B. (1947). Anaerobic fermentation of mannitol by staphylococci. J. Bact. 54 266.

EVANS, J.B. (1948). Studies of staphylococci with special reference to the coagulase - positive types. J. Bact. 55, 793.

EVANS, J.B., BRADFORD, W.L. and NIVEN, C.F. (1955). Comments concerning the taxonomy of the genera Micrococcus and Staphylococcus. Int. Bull. bact. Nomenol. Taxon. 5 61.

FORBES, D. and HEBERT, C.N. (1968). Studies in the pathogenesis of staphylococcal mastitis. Vet. Rec. 82 69.

FOUST, H.L. (1941). The surgical anatomy of the teat of the cow. J. Am. vet. med. Ass. 98 143.

FRANCK, L. (1875). - cited by Christiansen and Neilson, (1934).

FRASER, G. (1964). Studies of staphylococci of canine origin with particular reference to the nature and distribution of their haemolysins. Res. vet. Sci. 5 365.

GALL, A. Le and PLOMMET, M. (1965). Bacterial growth and leucocytosis in experimental staphylococcal mastitis in ewes. Annls Biol. anim. Biochim. Biophys. 5 113.

GALLI, A., GOLA, F. and RUFFO, G. (1967). Comportamento dei polinucleati neutrofili nella mastite bovina sostenuta da Staph. epidermidis Clinica vet., Milano 90 116.

GALLI, A. and GUALLINI, L. (1966). Diagnostic value of neutrophil polymorphonuclear cells in the milk of cows with mastitis. Archo vet. ital. 17 435.

GARVIE, E. I., HIGGS, T. M. and NEAVE, F. K. (1961). Unusual varieties of S. aureus. J. Path. Bact. 81 255.

GARRISON, E. R. and TURNER, C. W. (1936). The effect of udder irrigation and milking interval on milk secretion. Research Bull 234, Univ. of Missouri, Agric. exp. Station.

GIBSON, T. (1967). The status of the genus Micrococcus. Int. J. syst. Bact. 17 231.

GÖTZE, R. (1951). Zur Bekämpfung der Enterentzundugen des Rindes. Dt. tierärztl. Wschr. 58 213.

GORODETSKAYA, T. K. (1967). Changes in the processes of milk extraction in cows associated with physiological state of milk glands. Sb. nauch. Rab. vses. nauchno. issled. Inst. Zhivot. (4) 104.

GUALLINI, L. (1964). Early cytological diagnosis of mastitis. Atti Soc. ital. Sci. vet. 18 657.

HANSSEN, F. W. (1924). The bactericidal property of milk. Br. J. exp. Path. 5 271.

HARDING, H. A. and WILSON, J. K. (1913). A study of the udder flora of cows. N.Y. State agric. exp. Sta. Tech. Bull. 27

HASTINGS, E. G. and BEACH, B. A. (1937). The production of milk of abnormal composition by animals free from udder streptococci. J. agric. Res. 54 199.

HEIDRICK, H. J. and RENK, W. (1967). Diseases of the mammary glands of domestic animals. Sanders Co. Philadelphia and London.

HELMBOLDT, C.F., JUNGHERR, E.L. and PLASTRIDGE, W.N. (1953). The histopathology of bovine mastitis. Storrs Agric. exp. Station. Bull. 305.

HIBBITT, K.G. and COLE, C.B. (1968). The antibacterial activity of teat-canal cationic proteins. Biochem. J. 106 39p.

HICKMAN, C.G. (1964). Teat shape and size in relation to production characteristics and mastitis in a dairy herd. J. Dairy Sci. 47 777.

HIGGS, T.M., SMITH, A., CLEVERLY, L.M. and NEAVE, F.K. (1967). Corynebacterium ulcerans infections in a dairy herd. Vet. Rec. 81 34.

HOLMAN, H.H., PATTISON, I.H. and GORDON, W.S. (1952). Studies on experimental streptococcal mastitis VII. Immunological studies in goats. J. comp. Path. 62 196.

HOWELL, D.G., SMITH, I.M., HOLMAN, H.H. and PATTISON, I.H. (1956). Experimental streptococcal mastitis XI. Immunological studies in the cow. J. comp. Path. 66 49.

HOPKIRK, C.S.M. (1934). Consideration of the lactiferous duct in matitis of dairy cows. Aust. vet. J. 10 143.

HUBBEN, K., MORSE, G.E. and MEALEY, M.M. (1966). Studies of bovine mastitis. Histochemical observations on the streak canal epithelium. Cornell Vet. 56 648.

HUGHES, D.L. (1953). Experiments on methods to control the spread of Str. agalactiae in a dairy herd. Vet. Rec. 65 1.

HUGHES, D.L. (1960). Some epidemiological patterns in bovine mastitis. Vet. Rec. 72 485.

ILGMANN, G. (1933). Zitzenform und Striohkanalmündung in ihrer Beziehung zur Entstehung von Eutererkrankungen. Inaug. Diss. Hanover.

IMPERIAL BUREAU ANIMAL HEALTH, 1944. Modes of spread of Streptococcous agalactiae. Review Series No. 2.

INGALLS, W.L. and JOHNSON, E.P. (1947). The recovery of streptococci associated with bovine mastitis from the external surface of the teat. Am. J. vet. Res. 8 321.

JAHNKE, H-D. (1954). Beiträge zur Pathogenese und pathologischen Anatomie der Euterentzündungen unter besonderer Berücksichtigung der zu Melkbarkeitsstörungen führenden Veranderungen. Inaug. diss. Berlin.

JAIN, N.C. and JASPER, D.E. (1967a). Phagocytosis and destruction of Aerobacter aerogenes by leukocytes from bovine milk. Am. J. vet. Res. 28 405.

JAIN, N.C. and JASPER, D.E. (1967b). Viable cells in bovine milk. Br. vet. J. 123 57.

JAIN, N.C., JASPER, D.E. and CARROLL, E.J. (1967). Bactericidal activity for A. aerogenes of bovine serum and cell-free normal and mastitic milks. Am. J. vet. Res. 28. 397.

JAIN, N.C., LASMANIS, J. and SCHALM, O.W. (1967). Influence of egg albumin-induced leucopenia on experimental Aerobacter aerogenes mastitis and on natural infection of the mammary gland with coagulase-negative staphylococcus in a cow. Am. J. vet. Res. 28 1243.

JASPER, D.E. and JAIN, N.C. (1965). Some cultural characteristics of staphylococci isolated from milk. Can. J. comp.Med. 29 118.

JASPER, D.E. and JAIN, N.C. (1966). Haemolytic behaviour of staphylococci isolated from cows' milk. Can.J. comp.Med. 30 63.

JAYNE-WILLIAMS, D.J. and SKERMAN, T.M. (1966). Comparative studies on coryneform bacteria from milk and dairy sources. J. appl. Bact. 29 72.

JOHANSSON, I. (1957). Untersuchungen über die Variation in der Euter-und Strichform der Kühe. Z. Tierzücht. ZüchtBiol. 70. 233.

JOHNSTON, T. (1938). Anatomical and experimental study of the teat of the cow with particular reference to Streptococcal mastitis. J. comp. Path. 51 69.

JONES, G.W. and EDWARDS, S.J. (1966). Examination of an antibiotic produced by coagulase-negative staphylococci isolated from the bovine udder. J. Dairy Res. 33 271.

JONES, F.S. and LITTLE, R.B. (1927). The bactericidal property of cow's milk. J. exp. Med. 45. 319.

JONES, A. and RICHARDS, T. (1952). Night blue and victoria blue as indicators in lipolysis media. Proc. Soc. appl. Bact. 15 82.

JONES, F.S. and SIMMS, H.S. (1930). The bacterial growth inhibitor (Lactenin) of milk. 1. The preparation in concentrated form. J. exp. Med. 51 327.

KAMM, M. (1925). On the histological structure of the cow and goat teat. Inaug. Diss. Bern. Cited by Turner 1933.

KATSUBE, Y. and BLOBEL, H. (1964). *In vitro* phagocytic activities of leucocytes isolated from the mammary secretions of cows. Am. J. vet. Res. 25 1090.

KERR, W.R., PEARSON, J.K.L. and RANKIN, J.E.F. (1959). The bovine udder and its agglutinins. Br. vet. J. 115 105.

KITT, T. (1882). Relation between teats of domestic animals. Deut. Zeit. f. Tiermed. u. vergl. Path. 8 245. Cited by Turner 1933.

KLASTRUP, N.O. (1956). Clinical, biochemical and bacteriological effects of mastitis produced experimentally with staphylococci. Nord VetMed. 8 193-223.

KLIGMAN, A.M. (1963). The uses of sebum. Br. J. Derm. 75 307.

KLIGMAN, A.M. (1965). The bacteria of normal skin. In "Skin bacteria and their role in infection", by H.I. Maibach and G.H. Smith. McGraw Hill.

KOCUR, M., and MORTENSEN, N. (1967). Comparison of methods for estimation of anaerobic production of acid from glucose and mannitol in staphylococci and micrococci. Acta. path microbiol. scand. 71 141.

KOCUR, M., PRECECHTEL, F. and MARTINEC, T. (1966). Haemolysins in coagulase-negative staphylococci. J. Path. Bact. 92 331.

LASCELLES, A.K., OUTTERIDGE, P.M. and MACKENZIE, D.D.S. (1966). Local production of antibody by the lactating mammary gland following antigenic stimulation. Aust. J. exp. Biol. med. Sci. 44 169.

KREJAKOVIC-MILJKOVIC, V., SIPKA, M., JANKOVIC, Z., MARKOVIC, B. and SOVLJANSKI, B. (1967). Bovine mastitis: effects of teat shape and ease of milking (Title trans). Vet. Glasn. 21 719.

KRÜGER, W. (1953). Begünstigen bestimmte Zitzen und Enterformen die Ausbildung von Euterentzundüng beim Rinde? Tierärztl. Umsch. 8 432.

LITTLE, R.B. (1937). Bovine mastitis II. The production of mastitis by the suction of streptococci into the duct of the teat. Cornell Vet. 27 309.

McDONALD, J.S. and WITZEL, D.A. (1966). Differential pressures across the bovine teat canal during three methods of milk removal. J. Dairy Sci. 49 176.

McDONALD, J.S. and WITZEL, D.A. (1967). Teat sinus vacua at different milking machine vacua and pulsator ratios. J. Dairy Sci. 50 1237.

McDONALD, J.S. and WITZEL, D.A. (1968). Vacuum fluctuation at the teat end during mechanical milking. J. Dairy Sci. 51 543.

McEWAN, A.D. and COOPER, M.B. (1947). Bovine mastitis. Vet. Rec. 59 655.

McEWAN, A.D. and SAMUEL, J.McA. (1946). The experimental bacterial contamination of the inner structure of the teats and the milk of cows. Vet. Rec. 58 485.

McEWAN, A.D. and WHITE, M.B. (1950). Variations in the bactericidal and bacteriostatic properties of milk. Vet. Rec. 62 27.

MACKENZIE, D.D.S., OUTTERIDGE, P.M. and LASCELLES, A.K. (1966). Transfer of circulating Y-globulin into milk and efferent mammary lymph during experimental mastitis. Aust. J. exp. Biol. med. Sci. 44 181.

MANKOWSKI, H. (1903). The histological structure of the streak canal of the cow's teat. (Title trans) cited by Turner 1933.

MANUNTA, G., NUVOLE, P. and MARONGIU, A. (1966). Mungitura Meccanica e. Mastite nella vacca. Archo vet. ital. 17 51.

MARKHAM, N.P. and MARKHAM, J.G. (1966). Staphylococci in man and animals. J. comp. Path. 76 49.

MARKS, J. and VAUGHAN, A.C.T. (1950). Staphylococcal delta-haemolysin. J. Path. Bact. 62 597.

MARPLES, R.M. (1965). The effect of hydration on the bacterial flora. In Skin bacteria and their role in infection, by H.I. Maibach and G.H. Smith. McGraw-Hill.

MEYER-GOLLING, I. (1960). Histologische Untersuchungen der Zitzenkuppen bei leicht-und shwermelkbaren Kühen. Inaug. Diss. Berlin.

MILLER, W.T. and HEISHMAN, J.O. (1943). Staphylococcal antitoxin in the blood, milk and colostrum of cows. Am. J. vet. Res. 4 265.

MITCHELL, R.G. (1965). Staphylococci and urinary infection. Br. med. J. 1 1127.

MOCHRIE, R.D., HALE, H.H., EATON, H.D., ELLIOT, F.I. and PLASTRIDGE, W.N. (1953). Effects of vacuum level and milking duration on udder health in mastitis-free first calf heifers. J. Dairy Sci. 36 504.

MOCHRIE, R.D., HALE, H.H., DEMBICZAK, C.M., EATON, H.D., PLASTRIDGE, W.N., JOHNSON, R.E. and BEALL, G. (1955). Effects of vacuum level and milking duration on Guernseys and Holsteins differing with respect to lactation number and status of udder health. J. Dairy Sci. 38 1272.

MURPHY, J.M. (1944). The relationship of teat patency to udder infection. Cornell Vet. 34 64.

MURPHY, J.M. (1945). The relationship of teat mucous membrane topography to age, breed and incidence of udder infection in cows. Cornell Vet. 35 41.

MURPHY, J.M. (1959). The effect of certain mild stresses to the bovine teat canal on infection with Str. agalactiae. Cornell Vet. 49 411.

MURPHY, J.M. and STUART, O.M. (1955). Teat canal length in the bovine and its relation to susceptibility to swab-induced infection with Str. agalactiae. Cornell Vet. 45 112.

MURPHY, J.M. and STUART, O.M. (1952). Studies on the growth in vitro of a strain of Str. agalactiae in milk from infected and uninfected cows. Cornell Vet. 42 537.

MURPHY, J.M. and STUART, O.M. (1953a). The effect of introducing small numbers of Str. agalactiae (Cornell 48 strain) directly into the teat canal cavity. Cornell Vet. 43 290.

MURPHY, J.M. and STUART, O.M. (1953b). Some results of the application of Str. agalactiae (Cornell 48 strain) to the bovine teat canal by means of the Hadley-Wisconsin swab technique. Cornell Vet. 43 465.

MURPHY, J.M. and STUART, O.M. (1954a). The individual cow as a factor in Str. agalactiae infection artificially induced by means of the Hadley-Wisconsin swab technique. Cornell Vet. 44 268.

MURPHY, J.M. and STUART, O.M. (1954b). The swab exposure of cows to Str. agalactiae while protected from infection by means of penicillin. Cornell Vet. 44 349.

MURPHY, J.M. and STUART, O.M. (1954c). The syringe collection of milk via teat wall puncture as an aid in the study of artificial infection of the bovine mammary gland. Cornell Vet. 44 501.

MURPHY, J.M. and STUART, O.M. (1955). Machine milking rate and susceptibility to Str. agalactiae infection artificially induced by means of the swab technique. Cornell Vet. 45 262.

NEAVE, F.K., DODD, F.H. and HENRIQUES, E. (1950). Udder infections in the dry period. J. Dairy Res. 17 37.

NEAVE, F.K. and OLIVER, J. (1962). The relationship between the number of mastitis pathogens placed on the teats of dry cows, their survival and the amount of intra mammary infection caused. J. Dairy Res. 29 79.

NEAVE, F.K., OLIVER, J. and DODD, F.H. (1962). Effect of prolonged milking on the incidence of mastitis. 16th Int. Dairy Congr. A304.

NEAVE, F.K., SLOAN, J.K.B. and MATTICK, A.T.R. (1944). An outbreak of clinical mastitis involving 26 cows in a herd believed free from Str. agalactiae. Vet. Rec. 56 349.

NEWBOULD, F.H.S. (1964). A study of the penetration into the bovine mammary gland by Staph. aureus. Ph.D. Thesis, Reading.

NEWBOULD, F.H.S. (1967). Some effects of the source of bovine milk leucocytes and strain of staphylococcus on their interaction in vitro. Can. J. comp. Med. 31 303.

NEWBOULD, F.H.S. (1965). The stimulation by Tween 80 of a diphtheroid from cows' milk. Can. J. Microbiol. 11. 602.

NEWBOULD, F.H.S. and NEAVE, F.K. (1965a). The recovery of small numbers of S. aureus infused into the bovine teat cistern. J. Dairy Res. 32 157.

NEWBOULD, F.H.S. and NEAVE, F.K. (1965b). The response of the bovine mammary gland to an infusion of staphylococci. J. Dairy Res. 32 163.

NEWBOULD, F.H.S. and NEAVE, F.K. (1965c). The effect of inoculating the bovine teat duct with small numbers of S. aureus. J. Dairy Res. 32 171

NEWBOULD, F.H.S., SAURASTI, H.G. and BARNUM, D.A. (1966). Studies on phagocytosis of S. aureus by leucocytes from the bovine udder. I. Stimulation of staphylococci by milk leucocytes. J. comp. Path. 76 127.

NICOLAIDES, N. and WELLS, G.C. (1957). On the biogenesis of free fatty acids in human skin surface fat. J. invest. Derm. 29 423.

NOORLANDER, D.O. (1960). Milking machines and mastitis. Compton Press. California.

NOORLANDER, D.O. and SCHALM, O.W. (1958). A method for graphic measurement of the physical forces and differential pressures in mechanical milking systems. J. Am. vet. med. Ass. 133 474.

NORTON, J.F. and NOVY, (1932). Studies on the self-disinfecting power of the skin. Am. J. publ. Hlth. 21 1117.

NORTON, J.F. and NOVY, M.F. (1932). A further note on the disappearance of bacteria applied to the skin. Am. J. publ. Hlth. 22 193.

NYHAN, J.F. (1968). The effect of vacuum fluctuation on udder disease. Symposium on machine milking. Reading. (In press).

NYHAN, J.F. and COWHIG, M.J. (1967). Inadequate milking machine vacuum reserve and mastitis. Vet. Rec. 82 122.

OLIVER, J., DODD, F.H., NEAVE, F.K. and LEE, J.M. (1956a). Udder infections in the dry period II. The effect of withdrawing secretion from the dry udder on the incidence of mastitis. J. Dairy Res. 23 194.

OLIVER, J., DODD, F.H. and NEAVE, F.K. (1956b). Udder infections in the dry period IV. The relationship between the new infection rate in the early dry period and the daily milk yield at drying-off when lactation was ended by either intermittent or abrupt cessation of milking. J. Dairy Res. 23 204.

ORCUTT, M.L. and HOWE, P.E. (1922). Haemolytic action of a staphylococcus due to a fat-splitting enzyme. J. exp. Med. 35 409.

OUTTERIDGE, P.M. and LASCELLES, A.K. (1966). The cellular and immune response in milk and regional lymph during experimental mastitis. Res. vet. Sci. 7 360.

PAAPE, M.J. and TUCKER, H.A. (1965). Somatic cell content variations in fraction-collected milk. J. Dairy Sci. 48 801.

PETERSON, E.H., HASTINGS, E.G. and HADLEY, F.B. (1938). The pathology of non-specific mastitis and consideration of possible etiological agents. Cornell Vet. 28 307.

PETERSON, K.J. (1964). Teat injury resulting from improperly used milking machines. Sci. Proc. 101st Meet. Am. vet med. Ass. 32.

PETERSEN, W.E. (1944). The action of the mechanical milker in relation to completeness of milking and udder injury. J. Dairy Sci. 27 433.

PETERSEN, W.E. (1944). The action of the mechanical milker in relation to completeness of milking and udder injury. J. Dairy Sci. 27 433.

PHILLIPS, D.S.M. (1965a). The theory of milking action at the cow's teat: the mechanics of the cow's teat. Proc. of Milking Machine Summer School and Conference, p.34.

PHILLIPS, D.S.M. (1965b). Teat cup and claw: Mechanics of the teat cup. Proc. of Milking Machine Summer School and Conference, p.132.

PIER, A.C., SCHALM, O.W. and HAGE, T.J. (1956). A radiographic study of the effects of mechanical milking and machine vacuum on the teat structure of the bovine mammary glands. J. Am. vet. med. Ass. 129 347.

PIERCE, A.E. and FEINSTEIN, A. (1965). Biophysical and immunological studies on bovine immune globulins with evidence for selective transport within the mammary gland from maternal plasma to colostrum. Immunology 8 106.

PILLSBURY, D.M. and KLIGMAN, A.M. (1954). In "Modern Trends in Dermatology" ed. R.M.B. MacKenna. London: Butterworth.

PLASTRIDGE, W.N. (1958). Bovine Mastitis - A review. J. Dairy Sci. 41 1141.

POLLOCK, M.R., WAINWRIGHT, S.D. and MANSON, E.D. (1949). The presence of oleic acid-requiring diphtheroids on human skin. J. Path. Bact. 61 274.

POPHRISTOV, P. and TODOROV, A. (1965). White non pathogenic staphylococci as carriers of antagonistic properties of microbial flora over the skin, in the urethra and the mouth of man. Bulgarian Academy of Sciences. First Congress on Microbiology. p.279.

POUNDEN, W.D. and GROSSMAN, J.D. (1950). Wall structure and closing mechanisms of the bovine teat. Am. J. vet. Res. 11 349.

REBELL, G., PILLSBURY, D.M., PHALLE, M. de St. and GINSBURG, D. (1950). Factors effecting the rapid disappearance of bacteria placed on the normal skin. J. invest. Derm. 14 247.

RENK, W. (1967). Etiology, pathogenesis and morphology of bovine mastitis. Vet. med. Rev.(1) 3.

RICKETTS, C.R., SQUIRE, J.R. and TOPLEY, E. (1951). Human skin lipids with particular reference to the self-sterilising power of the skin. Clin. Sci. 10 80.

RUFFO, G. (1966). Research on the diagnosis of staphylococcal mastitis. Veterinaria ital. 17 627.

SANDVIK, O. and BROWN, R.W. (1965). Spectrophotometric characterisation of pigments produced by S. epidermidis strains isolated from bovine udders. J. Bact.. 89 1201.

SANDVIK, O. and FOSSUM, K. (1965). The classification of certain members of the family Micrococcaceae by serologic differentiation of theirproteolytic enzymes. Am. J. vet. Res. 26 357.

SCHALM, O.W., CARROLL, J. and LASMANIS, J.(1964). The leucocyte barrier and serologic investigations of experimental coliform mastitis in cattle. Am. J. vet. Res. 25 90:.

SCHALM, O.W. and LASMANIS, J. (1957). Distribution of micrococci and other bacteria in milk samples from a single dairy herd after twelve years of mastitis control. Am. J. vet. Res. 18 778.

SCHALM, O.W. and LASMANIS, J. (1963). Effects of a pre-existing leucocytic infiltration of the mammary gland upon experimental E. coli mastitis. 17th Int. Vet. Congr. 1 705.

SCHALM, O.W., LASMANIS, J. and CARROLL, E.J. (1964a). Pathogenesis of experimental coliform (Aerobacter aerogenes) mastitis in cattle. Am. J. vet. Res. 25 75.

SCHALM, O.W., LASMANIS, J. and CARROLL, E.J. (1964b). Effects of pre-existing leucocytosis on experimental coliform mastitis in cattle. Am. J. vet. Res. 25 83.

SCHALM, O.W., LASMANIS, J. and CARROLL, E.J. (1965). The use of a synthetic corticoid on experimental coliform (A.aerogenes) mastitis in cattle. The response of leucocytes and the effect of hormone induced neutrophilia. Am. J. vet. Res. 26 851.

SCHALM, O.W., LASMANIS, J. and CARROLL, E.J. (1965). Significance of leucocytic infiltration into the milk in experimental Str. agalactiae mastitis in cattle. J. Am. vet. med. Ass. 147. 1664.

SCHALM, O.W., LASMANIS, J. and CARROLL, E.J. (1966). Significance of leucocytic infiltration into the milk in experimental Streptococcus agalactiae mastitis in cattle. Am. J. vet. Res. 27 1537

SCHALM, O.W., LASMANIS, J. and CARROLL, E.J. (1967). Experimental Str. agalactiae mastitis in cattle: attempts to superimpose the organism in lactating glands harboring unrelated bacterial infections and in glands with experimentally induced sterile inflammation. Am. J. vet. Res. 28 685.

SCHALM. O.W., LASMANIS, J. and JAIN, N.C. (1967). Effects of humoral and cellular phases of acute inflammation in the bovine mammary gland on A. aerogenes introduced en masse and on leucocytes existing in milk. Am. J. vet.Res. 28 1251.

SCHALM, O. W., and WOODS, G.M. (1953). Micrococcus pyogenes in bovine milk II. Relationship of shedding characteristics to occurrence of clinical mastitis. Am. J. vet. Res. 14 534.

SCHEINMANN, L.G., KNOX, G., SHER, D. and ROTHMAN, S. (1960). The role of bacteria in the formation of free fatty acids on the human skin surface. J. invest. Derm. 34 171.

SCHIEMANN, O., and LANDAU, H. (1919). Über Handedes infektion und Handereinigung in ihrer Bedentung zur Verhütung von Krankheitsüberfragnungen. Z. Hyg. InfektKrankh. 88 129.

SCHMAHLSTIEG, R. (1957). Zur Konstitution des Euterdes Rindes 2. Morphologische Merkmale der Zitze beim schwarzbunten Rind. Dt.tierärtzl. Wschr. 64 157.

SCHMIDT, G.H., SWITZER, K.O., GUEST, R.W., and GUTHRIE, R.S. (1964). Effect of teat-end vacuum fluctuations on milking rate and mastitis. J. Dairy Sci. 47 761.

SHARPE, M.E., NEAVE, F.K., and REITER, B. (1962). Staphylococci and micrococci associated with dairying. J. appl. Bact. 25 403.

SLANETZ, L.W., BARTLEY, C.H., and ALLEN, F.E. (1959). The immunisation of dairy cattle against staphylococcal mastitis. J. Am. vet. med. Ass. 134 155.

SLANETZ, L.W., BARTLEY, C.H., and ALLEN, F.E. (1965). Evaluation of cell toxoid vaccines for the vaccination of dairy cattle against staphylococcic mastitis. Am. J. vet. Res. 26 688.

SMITH, J.W. and SCHULTZE, W.D. (1966). Variation in cell content of milk associated with time of sample collection. J. Dairy Sci. 49 738.

SMITH, J.W. and SCHULTZE, W.D. (1967). Variation in cell content of milk associated with time of sample collection. I. Diurnal variation. J. Dairy Sci. 50 1083.

SMITH, M.L. and PRICE, S.A. (1938a). Staphylococcus beta-haemolysin. J. Path.Bact. 47 361.

SMITH, M.L. and PRICE, S.A. (1938b). Staphylococcus gamma haemolysin. J. Path.Bact. 47 379.

SOLBERG, I. (1968). Streptococcus dysgalactiae; a possible disease - producing agent in various animal species. Nord. VetMed. 20 26.

SPENCER, G.R. and LASMANIS, J. (1952). Reservoirs of infection of Micrococcus pyogenes in bovine mastitis. Am. J. vet. Res. 13 500.

STABENFELDT, G.H. and SPENCER, G.R. (1966). The lesions in bovine udders shedding non-haemolytic coagulase-negative staphylococci. Pathologia vet. 3 27.

STANLEY, D.E., KESLER, E.M. and BORTREE, A.L. (1962). Effect of a fluctuating vacuum on certain measures of udder health. J. Dairy Sci. 45 1343.

STUART, P. and LANCASTER, J.E. (1949). Some factors which may be concerned in the susceptibility of the bovine udder to Str. agalactiae infection. J. comp. Path. 59 31.

SUBCOMMITTEE ON TAXONOMY OF STAPHYLOCOCCI AND MICROCOCCI (1965). Int. Bull. bact. Nomencl. Taxon. 15 109.

SWEENEY, E.J. (1964). Observations on the epidemiology of mastitis due to Str. uberis in a laboratory herd during a complete lactation. Res. vet. Sci. 5 483.

TAYLOR, J.I. (1949). Some aspects of the epidémiology, bacteriology and pathology of bovine mastitis. Ph.D. Thesis. Reading University.

TERPLAN, G. and GEDEK, W. (1965). Zur Differenzierung von aus Rinderanten isoliten Micrococcaceae. Archiv Lebensmittelhyg. 16 203.

THIEL, C.C. (1968). Machine factors affecting milking performance: Forces acting on the teat. Symposium on Machine Milking. Reading. (In press).

THIEL, C.C., CLOUGH, P.A. and DODD, F.H. (1965). Vacuum inside the cow's teat at end of machine milking. J. Dairy Sci. 48 617.

THIEL, C.C., CLOUGH, P.A., WESTGARTH, D.R. and AKAM, D.N. (1966). Mechanics of machine milking II. The flow-rate pattern within single pulsation cycles. J. Dairy Res. 33 177.

TREECE, J.M. and MORSE, G.E. (1965). Bovine mastitis III. Composition of lipids of teat canal soft keratin. J. Dairy Sci. 48 821.

TREECE, J.M., MORSE, G.E. and SHAH, P.C. (1964). Basic studies of bovine mastitis II. Chemical composition of teat canal soft keratin. J. Dairy Sci. 47 697.

TREECE, J.M., MORSE, G.E. and LEVY, C. (1966). Lipid analysis of bovine teat canal keratin. J. Dairy Sci. 49 1240.

TURNER, C.W. (1930). The anatomy of the mammary gland of cattle. Embryonic Development. Univ. of Missouri Agr. exp. St. Bull. Research. 140. 1.

TURNER, C.W. (1933). The anatomy of the mammary gland of domestic animals and laboratory animals. College of Agriculture, Univ. of Missouri.

UDALL, D.H., JOHNSON, S.D. and FERGUSON, J. (1938). The control of mastitis in New York State. Vet. Rec. 50 1417.

VENZKE, C.E. (1940). A histological study of the teat and gland cistern of the bovine mammary gland. J. Am. vet. med. Ass. 96 170.

WAITE, R. and BLACKBURN, P.S. (1957). The chemical composition and the cell count of milk. J. Dairy Res. 24 328.

WAITE, R. and BLACKBURN, P.S. (1963). The relationship between milk yield, composition and tissue damage in a case of subclinical mastitis. J. Dairy Res. 30 23.

WALSER, K. (1966). Melkmaschine und mastitis. Paul Parey, Berlin, Hamburg.

WARD, A.R. (1900). The invasion of the udder by bacteria. New York (Cornell) Agric. exp. Sta. Bull. 178.

WEBER, A.F., WYAND, D.S. and PHILLIPS, M.G. (1957). Studies of the incidence and morphology of accessory glandular tissue in the teat canal of the bovine mammary gland. Am. J. vet. Res. 18 761.

WHITE, F. and RATTRAY, E.A.S. (1965). Diurnal variation in the cell content of cows' milk. J. comp. Path. 75 253.

WHITTLESTONE, W.G. and OLNEY, G.R. (1962). Machine milking and mastitis 3. Some properties of the bovine teat sphincter under machine milking conditions. Aust. J. Dairy Technol. 17 205.

WILLOUGHBY, R.A. (1966). Bovine staphylococcic mastitis. An immuno histochemical study of the cellular sites of antibody formation. Am. J. vet. Res. 27 522.

WILSSENS, A.T.E., VANDE CASTEELE, J.C. and DEMETER, R.L. (1966). Staphylococci and Micrococci in raw milk. 17th Int. Dairy Congr. B 431.

WILSON, A.T. and ROSENBLUM, H. (1952a). The antistreptococcal property of milk I. Some characteristics of the activity of lactenin in vitro. The effect of lactenin on haemolytic streptococci of several serologic groups. J. exp. Med. 95 25.

WILSON, A.T. and ROSENBLUM, H. (1952b). The antistreptococcal property of milk. III. The role of lactenin in milk-borne epidemics. The in vivo action of lactenin. J. exp. Med. 95 51.

WILSON, C.D. (1958a). IN Rep. An. Hlth Div. (1958). M.A.F.F., H.M.S.O.

WILSON, C.D. (1958b). Factors that predispose to mastitis with special reference to milking technique. Vet. Rec. 70 159.

WILSON, C.D. (1968). Personal communication.

WILSON, C.D. and BROCKBANKS, E.O. (1967). The limited value of herd sampling in the control of bovine mastitis in New Zealand. N.Z. vet. J. 15 109.

WINTON, F.R. and BAYLISS, L.E. (1955). Human Physiology. 4th ed. p.353.

WITZEL, D.A. (1965). Rhythmic contractions of the teat sphincter in the bovine. J. Dairy Sci. 48 251.

WITZEL, D.A. and McDonald, J.S. (1964). Bovine intramammary pressure changes during mechanical milking. J. Dairy Sci. 47 1378.

WITZEL, D.A. and McDonald, J.S. (1965). Intramammary pressures between milkings in the lactating cow. J. Dairy Sci. 48 726.

ZLOTNIK, I. (1947). Types of cells present in cow's milk. J. comp. Path. 57 196.

Abstract

The infections present in teat canals during two lactations of a herd of dairy cows have been investigated. Special attention was given to Micrococcaceae which were commonly isolated from swabs inserted 3mm into the teat canals. The taxonomy of the bacteria was determined by Baird-Parker's (1963) classification. Cultures of all his subgroups were identified and the proportion of each varied throughout the periods of examination: some types were rarely isolated. In addition cultures were found with different reactions to those described by Baird-Parker. Accurate identification of the bacteria enabled their epidemiology to be examined in detail and the results during one lactation are reported. The distributions of the bacterial subgroups are presented in relation to the standing positions of the cows in the byre during milking. Bacteria which were increasing in incidence were usually isolated from one or two cows originally. The infections then spread to the nearest cows laterally and across the byre and was independent of the milking routines.

The distribution of cultures with similar reactions provided evidence of taxonomical similarities between some of Baird-Parker's subgroups and cultures with atypical characters. The results showed also that the Micrococcaceae were able to survive long periods within the teat canal. This was found to be true for teat canal keratin in vitro. Keratin was collected from the teat canals of other animals and the ability of the natural bacterial population to survive

determined in conditions which simulated the teat canal environment. The Micrococcaceae subgroups which were isolated from the material survived for at least 27 days and some were viable after 60 days incubation. The methods of ..culturing from the specimens did not always isolate bacteria shown to be present in subsequent subcultures and it is suggested that bacteria do not permeate through the material unless mechanically assisted. The ability of keratin to retain bacteria *in vitro* was often paralleled by the isolation of the same bacterium from the concomitant teat canal at similar periods after collection. In another lactation the rôle of teat canal infections in bacterial invasion of the mammary parenchyma was investigated. New intramammary infections of Micrococcaceae and Corynebacterium bovis recognised by collection of milk by syringe through the teat wall confirmed in some cases the bacteria were localised some weeks in the teat canal before becoming established in the gland itself. The pathogenic significance of various intramammary infections was assessed on the basis of elevated cell counts in foremilk. C. bovis and coagulase negative staphylococci both caused elevated cell counts to the extent that it was suspected that their presence rendered glands relatively resistant to invasion by other more pathogenic bacteria present in the teat canals.

A further experiment showed that the bacteria in the teat canal are not refluxed into the residual milk in the teat sinus by correctly conducted milking with an efficient machine.

It is concluded that the pathogenesis of infection of the mammary gland parenchyma by the passage of bacteria through the teat canal may be a process of some weeks duration. The mechanics of machine milking may assist the carriage of bacteria resident in the keratin along the walls of the teat canal to reach the teat simus.