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A Critical Examination of the Presumptive Coliform Test in Milk

by

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A Critical Examination of the Coliform Test in Milk

Preface

The Presumptive Coliform test is used as an indicator of possible faecal contamination of water supplies. In the attempts to provide a bacteriological standard for milk of good keeping quality which is safe for human consumption, the same test has been applied to milk. Thus, milks of high coliform content tend to be regarded by Department of Health authorities as "not up to standard" and not suitable for marketing. This view antagonises the farmer or producer who suffers financially if his milk is withheld from the market. His immediate reaction is to condemn the coliform test as valueless because it is not a true indicator of the standard of milk.

The controversy arises because, for water samples, the presumptive coliform test is a good indicator of faecal contamination but has quite a different significance in milk. A positive test with milk may indeed indicate faecal contamination but there are many other possible explanations. Above all coliforms quickly die in water and their presence therefore shows recent faecal contamination; whereas in milk the organisms not only do not die but grow abundantly. Opinion differs greatly on the validity of the test, as can be seen from the fact that in England the test was abolished in 1949 whereas it is still retained in Scotland. In America it is retained for milk of "Certified" grade.

The chief aim in milk production should be to provide the consumer with milk of good keeping quality and free from any pathogenic bacteria. How does the presumptive coliform test stand in this matter? In Scotland there are three main grades of milk namely, "Certified", Tuberculin Tested (T.T.) and Pasteurised milk, and /

and each has its own coliform-test standard. "Certified" milk comes from tuberculin-tested herds, is produced under very clean conditions, and is bottled on the farm. It is regarded by many people as the "best" milk. T.T. milk comes from tuberculin-tested herds and may or may not be pasteurised. In Scotland 99.8% of herds are T.T. (Chalmers and Sampson, 1958). Pasteurised milk has undergone heat treatment to destroy any pathogenic bacteria which may be present. Undoubtedly it will be the safest milk, but many people dislike its 'flat' taste and are under the impression that it loses some important fraction of its nutritive value after heat treatment.

With the above points in view, it was decided to examine milks of different grades, at different seasons, as purchased by the consumer i.e. milk delivered in bottles after it had passed through all stages of production. This was decided upon because most routine testing of milk is done before the milk reaches the consumer, usually on individual samples from farms or on bulk samples at creameries. It seemed of interest to discover whether the bacteriology of the milk would shed some light on the possible status of the test by the time that the milk had actually reached its destination. Presumptive coliform tests were carried out, and positive samples were further examined to see whether coliform organisms were indeed responsible for the positive tests. Samples were also examined by other tests and some interesting points arose. One of the most surprising was the high incidence of Staphylococcus aureus in raw milk, and the revealing fact that on bacteriological grounds "Certified" milk is far from being a "good" milk compared with Pasteurised milk.

The following work is therefore directed mainly towards findings on the examination of milks by the presumptive coliform test. It also includes, however, a number of relevant observations on the significant bacterial flora of consumer milk.

Introduction and Review of the Literature

The Presumptive Coliform test

In water analysis the coliform test is a good index of faecal pollution. Coliform organisms, especially Escherichia coli, are found in human and animal intestines, and their presence in water is an indication that other intestinal pathogens can gain access to water. Because coliform organisms tend to die out rapidly in water, the test is also an index of recent faecal pollution. However, saprophytic non-pathogenic, coliform organisms e.g. Klebsiella aerogenes may also be present in water, in soil, and on vegetation and these may be responsible for positive coliform tests as the presumptive method is statutorily performed.

The Presumptive Coliform test in water is based on the fact that coliform organisms will grow in a medium containing bile salt and lactose, and will ferment lactose with the production of acid and gas. The test consists of inoculating different volumes of water into tubes containing such a medium e.g. MacConkey's bile salt broth. A small Durham tube is included to show production of gas. There is a large sampling error, therefore several tubes are set up for each dilution of water. The number of coliform organisms present in the water sample can then be found from probability tables devised by McCrady (1918). The test is used in standard water analysis (The Bacteriological Examination of Water Supplies, 1956).

The routine examination of milk includes a test which is a modification of the presumptive coliform test for water. If milk is produced in good clean surroundings and comes from healthy cows, coliform organisms should nearly always be absent. Presence of coliform organisms affects the keeping quality of the milk and indicates either faecal pollution or unclean milking utensils. It would /

would seem therefore that any test which shows the presence of a large number of coliform organisms in milk, would be desirable as a means of maintaining a good standard of dairying and of producing milk of good keeping quality. For these reasons the Presumptive Coliform Test is a routine test in Scotland and for certain grades of milk in America. Because a large number of milks fail to pass this test, farmers have claimed that it is valueless because it condemns many milks suitable for consumption. Because coliforms are not normally pathogenic and a positive test can be false, farmers would prefer the test to be dropped completely. In England this has happened and the coliform test was abandoned. This matter is being discussed by the Department of Health for Scotland (1959), which wonders whether the time has come to revise the bacteriological control of milk supplies. The general opinion seems to be that the coliform test is time-consuming and does not measure the hygienic or keeping quality of milk. I do not myself think that its abandonment is justified when there is evidence available (Brodie, 1959), that many milks contain coliforms, often of faecal varieties, indicating poor production methods and possibly poor keeping quality of the product. The standard of milk in Scotland is regarded as high, but even so, there is a high incidence of positive coliform tests. I think it would be a retrograde step to abandon the test. It is not too rigorous - a farmer must have three consecutive failures at intervals of 14 days before any action is taken by the authorities. The fact remains that although it is not necessarily an indication of faecal pollution in milk or of the presence of pathogenic organisms, the presumptive coliform test is a good check on the cleanliness of milking methods, production, and bottling, and gives an indication of the keeping quality of milk.

Positive tests are given by coliform organisms which can grow in the presence of bile salts and ferment lactose present in the medium /

medium to give acid and gas. Other organisms, acting synergistically, may also produce acid and gas and give false positives. In the examination of milk, 1 ml. quantities of dilutions of milk are added to tubes of MacConkey broth in triplicate and incubated at 37°C for two days. The fact that the results are not read till after 48 hr. is also another argument against the test. Results are expressed as the presence or absence of presumed coliforms (= acid and gas in the test) in a given volume of milk e.g. 0.1 ml. No attempt is made to record actual numbers (cf. water analysis).

The significance of the test differs for water and milk. In the examination of water, the presence of coliform organisms (and especially E.coli) is an indication of faecal pollution. The same can apply to milk but there are other possible sources of the organisms e.g. milking utensils, hay, straw, animals' coats, water supply and dirty bottles. Thus a positive test in milk cannot be regarded as being solely due to faecal pollution, though that possibility is not excluded. In both cases, a further investigation of the positive test is necessary to ascertain whether the organisms are faecal (Escherichia) type or saprophytic (Klebsiella and Intermediate) type.

Coliform Organisms

For the purpose of this work, coliform organisms will be regarded as the small Gram-negative, non-sporing, rods of the genera Escherichia and Klebsiella, together with Intermediate types which may be saprophytic or found in the intestines of man and animals. The genus Escherichia is rarely found outside the intestine but Klebsiella and Intermediate types may occur saprophytically in soil and water and on plants and grasses.

Coliforms may be isolated on selective media containing bile salt which suppresses most non-intestinal organisms. The medium most /

most often employed is MacConkey's bile salt, lactose medium containing an indicator which will show fermentation of lactose. The presumptive coliform test is carried out in liquid media and several such have been employed by different workers in an attempt to give better and quicker results, to allow equal growth of all types of coliforms, and to avoid false positives given by organisms other than coliforms.

Media used in the Presumptive Test

Horwood and Heinfetz (1934) compared a series of media and found that standard lactose broth was the most sensitive indicator of coliforms. The other media studied - Salle's crystal violet lactose broth, Dominick and Lauter's broth and Jordan's brilliant green, lactose, bile, peptone broth weakened or destroyed coliforms with the long period of incubation. A similar survey was made by Black and Klinger (1936) who also found that standard lactose broth was the best medium and in agreement with Horwood and Heinfetz, obtained good results with brilliant green lactose bile broth.

Atkinson and Wood (1938A), studying water supplies in Victoria, Australia, attempted to find a more suitable medium because they were troubled by large numbers of false-positive results. Of the 4 media studied - MacConkey broth, lactose broth plus indicator, Salle's crystal violet broth, and a synthetic medium containing lactose, asparagine and brom-cresol purple, they, too, found MacConkey broth to be the most satisfactory. Lauryl sulphate broth has been used by Dyett (1957) to detect the presence of E.coli in ice cream. The broth has no indicator and has a tendency to foam, making early detection of fermentation difficult. The author claims quicker detection of E.coli because indole and Eijkman tests may be carried out directly and simultaneously with this medium. On the whole it is agreed that MacConkey's lactose, bile salt broth is the most suitable medium to use. It has been suggested (Druce et al., 1957) that a colony /

colony count on violet red bile agar, incubated at 30°C, is as good an indicator of coliform organisms in milk as MacConkey broth. This avoids overgrowing of one type by another, and cuts out the high sampling error of the dilution method. However this method is not entirely selective for coliforms. Griffith et al. (1956) who used a similar method, but with Levine's eosin-methylene blue agar, found that only two-thirds of the characteristic colonies were true coliforms. They concluded that the method was too unselective for routine use.

The time and temperature of incubation is also open to discussion. The standard methods of water analysis and milk-testing require 48 hours' incubation at 37°C. Some strains of K.aerogenes common in milk, grow very slowly at 37°C and grow well at 30°C and it is sometimes advised to incubate at 30°C for 72 hours (Chalmers, 1955). Murray (1953) carried out a survey of the comparison of 30°C and 37°C incubation temperatures in the test for raw and pasteurised milk. He found that 61 cultures isolated from tubes which were positive at 30°C but negative at 37°C (even when retested) grew well on yeastrel agar slopes at 37°C. He concluded the limiting factor of growth at 30°C was the combination of MacConkey medium and temperature, and not temperature alone, because the cultures grew freely at 37°C on other media. He suggested that incubation at 30°C rather than 37°C would be a much better indicator of hygienic quality of milk. Another disadvantage of carrying out the test in liquid medium is that one organism may outgrow the other and in the higher dilutions a differential test may show types which bear no relationship to those which were present originally (Wilson et al.,1935). Other factors may influence the test. Anderson (1957) has shown that certain types of peptone when used in the medium may fail to show positive tests. If the medium is suitably buffered this effect is /

is reduced to some extent.

The accuracy of the test has been examined by Barkworth and Irwin (1938). Owing to the physico-chemical structure of milk it was thought that coliform organisms might have a tendency to clump, thereby causing error in higher dilutions. They found that the distribution of organisms is accurate enough to give a good idea of the numbers of bacteria present per ml. On the other hand the same authors (Barkworth and Irwin, 1943-44) later described the coliform test sometimes underestimating the coliform population of milk. Known numbers of coliforms were added to milk and water and tests carried out in parallel. Milk gave a lower proportion of positives and it was suggested that this was due either to a physical reason e.g. clumping of the organism and association with fat globules, or failure of the organism to grow, due to a bactericidal action of the milk or medium. Bile salts were tested for their bactericidal power and were found to have no effect on coliforms.

Very often false-positive reactions in media used are caused by organisms other than coliforms. Salle (1930) described false positive tests due to three different causes viz. (1) anaerobic organisms, (2) synergistic reactions between two different organisms, and (3) sporing aerobes. Atkinson and Wood (1938B) described false positives due to Clostridium welchii in association with a Proteus species, and to a Proteus species in conjunction with E.coli-anaerogenes or Streptococcus faecalis.

In the present work I have sought to verify that the organisms responsible for positive tests were indeed coliforms and to identify others which were not.

Coliforms in Milk

Milk produced and handled under good hygienic conditions should contain very few coli-aerogenes bacteria. Presence of such organisms /

organisms in large numbers is evidence of carelessness at some stage during production, e.g. insufficient cooling after production or during storage. The presumptive coliform test for milk is not a statutory test in all countries because it does not have the same significance as it does for water supplies. In U.S.A. it is used only for "Certified" milk and in Scotland it has been a statutory test for the past 33 years. In England however, it was abandoned in 1949 because it showed a high proportion of failures in winter (Thomas, 1955). The test is of value in the control of milk supplies. Malcolm (1930) states that "it is probably the most valuable test for drawing the line between 'clean' and 'dirty' milk". The presence of large numbers of coliforms may indicate manurial contamination, unclean utensils or milking machines, or a contaminated water supply, and will affect the keeping quality of the milk.

There is a seasonal variation in the coliform content of milk (Malcolm, 1930, 1934, 1939). There is also a variation in the proportion of the types of coliforms isolated. In summer, Malcolm (1933) found that the ratio of E.coli: A.aerogenes was 0.7/1 and in winter it was 2.4/1. He found that in winter 71% of coliforms isolated were E.coli and 7.5% were A.aerogenes compared with 40.4% E.coli and 22.4% A.aerogenes in summer. Malcolm (1939) suggested that the high incidence of E.coli during the winter months was due to the fact that the animals are kept more in the byre, where there are more opportunities for faecal contamination, than when the cows are out at pasture. The higher incidence of positive presumptive tests in summer, and the fact that many are due to the aerogenes-cloaceae group, may be explained by the fact that even a small number of these organisms in milking machines and utensils may become greatly increased by the favourable temperatures (17°C) of milk /

milk during that period. Indeed, as already mentioned (Chalmers, 1955), lower temperatures have been suggested for incubation of the coliform test.

Stuart et al. (1938) found a predominance of Aerobacter and Intermediate types of coliforms among their isolates from "Certified" milk, and suggested that the test was of doubtful value. Chalmers (1928) concluded that the coliform test could not be relied upon to give a true indication of the presence of coliforms in milk. He found that 65.7% of coliforms isolated from 268 samples of "Certified" milk were true E.coli.

The coli-aerogenes bacteria of pasteurised milk are also subject to seasonal variation (Papavasseliou, 1957). True E.coli was found in the months June-August but not during the winter, when the predominating bacteria were Klebsiella species and Citrobacter freundii. As none of the organisms were resistant to the temperatures of pasteurisation, contamination must have followed pasteurisation. It has been shown (Beavens, 1930) that lactose (present in milk in 4.5% concentration), has a protective action on E.coli heated at pasteurisation temperatures. The greater the concentration of lactose, the greater is the possibility of survival of the organisms. In Scotland (1957) 2.7% of 5,719 pasteurised milk samples failed the coliform test. During the warmer months, 5.8% failed, whereas in winter only 0.6% failed (Chalmers and Sampson, 1958). Unpasteurised milks, e.g. "Certified" and T.T. had the following coliform failures:-

"Certified" 21.5% T.T. 26%

The fact that milk provides a most suitable medium for bacterial multiplication explains the higher incidence of positive tests during the summer months and emphasises the need for adequate cooling of milk after production.

Classification /

Classification of Coliforms

The "coliform" bacteria belong to the tribe Escherichieae in the family Enterobacteriaceae. Further subdivision into genera and species has been the subject of much controversy. The organisms are all Gram-negative rods which ferment lactose with the production of acid and gas, and may be motile or non-motile. MacConkey (1905) described the lactose-fermenting, Gram-negative rods in faeces and divided them mainly on sugar fermentations, production of indole, motility, Voges-Proskauer reaction and liquefaction of gelatin. At this time the generic name given to such organisms was Bacterium (or even Bacillus); thus we have B.coli and B.lactis-aerogenes, both belonging to the same group in MacConkey's classification. Levine (1917) showed that the methyl red and Voges-Proskauer tests could be used to separate Escherichia coli from Aerobacter aerogenes, these being two distinct species belonging to different groups. The present classification in Bergey's Manual (1957) has not altered from that of the previous edition (1948) and still divides the Escherichieae into 3 main genera viz.:-
Escherichia consisting of mainly intestinal organisms.
Aerobacter which may be found in faeces but more often in milk, dairy products, grain and saprophytic situations.
Klebsiella, very similar to Aerobacter but found mainly in respiratory tract. Also included are the "paracolon" bacteria which show delayed or absent lactose fermentation.

The differences between faecal Escherichia types and saprophytic Aerobacter types is quite obvious. The trouble arises when trying to decide where the numerous Intermediate coliforms belong. These organisms have properties midway between Escherichia and Aerobacter and in the examination of water supplies it may be difficult to decide whether faecal contamination has taken place when only Intermediate /

Intermediate types are isolated. Bergey has called citrate-positive E.coli-like organisms E.freundii. The latest classification by the Enterobacteriaceae Sub-Committee (1958) has placed them in a separate genus Citrobacter.

Again difficulties arise when trying to separate Aerobacter cloacae from Klebsiella. Recent work shows that Klebsiella can be separated from Aerobacter cloacae by examination of the decarboxylase enzymes present. Klebsiella organisms contain lysine decarboxylase and no ornithine decarboxylase whereas cloacae organisms have weak lysine and strong ornithine decarboxylases (Møller, 1954, Ørskov, 1955) Cowan (1956) suggested that A.aerogenes should be included in the genus Klebsiella because its biochemical and serological properties are indistinguishable from those of Klebsiella (Edwards and Fife, 1955) and be given the name K.aerogenes because the name Klebsiella has priority over Aerobacter. A.cloacae should be placed in a separate genus Cloaca, because it is motile, liquefies gelatin and has a different amino acid decarboxylase system. Citrate-positive, E.coli-like, organisms are placed in a separate genus, Citrobacter.

Kauffman (1951) described a serological classification of Escherichia and related organisms, similar to the Kauffmann-White scheme for the Salmonella. Certain of the E.coli serotypes (which do not differ biochemically from other E.coli) are now associated with outbreaks of infantile diarrhoea. It is possible that these types might cause infection in babies if they were present in large numbers in milk. Thomson (1956) surveyed raw milk arriving at pasteurising plants and found that 1% of samples contained pathogenic serotypes of E.coli. He suggested that the reservoir of infection might be farm poultry, but it would seem more likely to be associated with organisms causing "scour" in calves. This is confirmed by Rees (1957) who recovered two antigenic types of E.coli previously /

previously known to occur only in epidemic infantile gastro-enteritis, from outbreaks of "scour" in calves. Papavasseliou (1957) examined pasteurised milk for pathogenic serotypes of E.coli and although 101 of the 375 samples examined contained coliforms, these serotypes were not found.

A positive presumptive coliform test may be given by one or more than one of the coliforms already described. In water examination the problem arises whether they are of faecal origin or not. Further differential tests are necessary. In milk, however, the mere presence of any type of coliform is significant, and the presence of large numbers of any such organisms indicates faulty handling of the milk. The difficulty with milk is that there are numerous sources from which they can gain entrance to milk and multiply rapidly, as milk is a better nutrient medium than water. In addition, false-positive tests may depend on synergistic action between two or more different bacteria. This is even more likely in milk which contains much protein and other substrates for bacterial enzymes.

Classification of Coliforms Found in Milk

As a result of the numerous attempts to classify coliform bacteria by various biochemical tests, Malcolm (1938) attempted to form a guide to classification of coliform organisms isolated from milk and bovine faeces. He was of the opinion that the coliform group resembled a continuous spectrum of closely related species and that there was little justification for subdivision into 2 or more genera (cf. Bergey). He preferred to divide the organisms into 8 subgroups by the original criteria of MacConkey (1905, 1909) already mentioned with the addition of Koser's citrate reaction and fermentation of inositol. The eight subgroups may be differentiated by 4 main criteria viz. Voges-Proskauer, Koser, inositol and indole reactions (Fig. 1).

Voges- /

Voges-Proskauer reaction - production of acetyl-methyl-carbinol from glucose

Koser reaction - ability to use sodium citrate as sole source of carbon, when the sole source of nitrogen is inorganic

indole reaction - production of indole from tryptophane

inositol - fermentation of the sugar

Fig. 1

Malcolm's classification of coliform organisms isolated from milk and bovine faeces

Subgroup of types	V.P.	Koser	Inositol	Indole	Habitat
1. B.coli	-	-	-	+	Most prevalent in intestine and faeces
2. Intermediate	-	+	-	+	Found occasionally in soil and faeces
3. Intermediate	-	+	-	-	Found frequently in soil, occasionally in faeces
4. Intermediate	-	+	+	+	Seldom found in either soil or faeces
5. B.friedlanderi	-	+	+	-	Found in upper respiratory tract and to a limited extent in faeces
6. B.cloacae *	+	+	-	-	Found in intestine, faeces, soil and on plants
7. B.oxytocus *	+	+	+	+	Found occasionally in faeces
8. B.aerogenes	+	+	+	-	Found frequently in faeces, soil and on plants

* Frequently liquefy gelatin

The /

The most important point which emerges from this is the striking difference between groups 1 and 8. Those would appear to correspond to the two type species of Bergey's classification into genera viz.

Escherichia coli Type I (cf. Malcolm, group 1) and Aerobacter aerogenes (cf. Malcolm, group 8). The classification of other species by Bergey is not satisfactory but in accordance with Malcolm's and other classifications it would appear that we can recognise two distinct species or subgroups

(1) Those identical with the "typical" E.coli and found most prevalently in the intestine, faeces, and sewage, and especially in bovine faeces (Malcolm, 1935)

(2) Those identical with K.aerogenes, found to some extent in bovine faeces but more prevalent in soil, water and on plants.

There is a wide range of organisms with properties intermediate between these two types. With regard to the above classifications, I decided, when identifying coliform organisms in this work, to determine whether they were typical E.coli or K.aerogenes. Other organisms possessing the general characteristics of the coliform group, but intermediate in their properties between E.coli and K.aerogenes will be referred to as Intermediate coliforms including Citrobacter species. Organisms which have the general characteristics, but which ferment lactose weakly or after an interval will be regarded as paracolon types.

Coliform organisms referred to in this work are Gram-negative, non-sporing, aerobic but facultatively anaerobic, motile, or non-motile rods fermenting lactose to give acid and gas and capable of growing in the presence of bile salt. Two main species will be recognized viz.

Escherichia /

Escherichia coli - when fermenting carbohydrates, produces carbon dioxide and hydrogen in the ratio 1 to 1 and gives a high acidity i.e. about pH 4.5 i.e. positive methyl red test. Indole reaction is positive, Voges-Proskauer and Koser citrate reactions are negative. In addition these organisms will ferment lactose to give acid and gas when incubated at 44°C. This test was originally introduced by Eijkman (1914) and had an incubation temperature of 46°C. Results were very variable until it was found that success depended upon maintaining the temperature of the medium between 43° and 45°C (Wilson et al., 1935).

Klebsiella aerogenes - organisms when fermenting carbohydrates produce carbon dioxide and hydrogen in the ratio 2 to 1 and do not reach a high acidity i.e. methyl red test is negative. They do not form indole but give positive Voges-Proskauer and Koser reactions. Lactose is not fermented at 44°C. These organisms may have marked capsules.

Both organisms will form lactose positive, smooth colonies on MacConkey's agar at 37°C. Some Klebsiella cultures may not grow at 37°C but will grow at 30°C.

Both organisms may be isolated from tubes of fermented MacConkey medium by plating on to MacConkey agar and incubating at 37°C. As already mentioned some Klebsiella species may fail to grow. For differential coliform estimations, in order not to miss detection of some types, modification of this technique (Wilson et al., 1935) has been suggested. This involves inoculating a MacConkey medium to be incubated at 44°C and a Koser citrate medium, to be incubated at 37°C. In this way there is less chance of K.aerogenes being missed and reported as being absent.

Scheme of Work

Many /

Many surveys have been made of the presumptive coliform test in milk and of the seasonal distribution of the coliforms isolated. The milk samples studied are usually taken directly from farms or from large tanks at creameries. It was decided in the present work to take samples of milk from bottles as sold to the consumer, and to study them throughout the year. Several grades of milk were examined - "Certified" milk, Tuberculin-tested and Pasteurised. The main object was to determine the incidence of positive coliform tests and to isolate the organisms responsible for the positive reactions. In addition plate counts at 37°C were carried out for comparison. During the work I noticed that many of the plates had large numbers of Staphylococcus aureus colonies. This was investigated further, bearing in mind the possibility of Staph.aureus food poisoning in young children who might consume large quantities of such milk. The results and conclusions of this investigation are described in Section II. Milk was also examined for the presence of Clostridium welchii. This test is sometimes used as an indication of faecal contamination of water. It is not suggested that this should be used as a test for faecal contamination of milk, because farms, surrounding buildings, and soil must harbour many spores of this organism. It was intended to correlate this with the types of organisms isolated in the presumptive tests. Cl.welchii is a possible source of false positives in the presumptive coliform test (Atkinson and Wood, 1938B) and hence it was desirable to know if it was present in milk samples giving such results. During the investigation it was found that "stormy clots" are very often given by other organisms, or combinations of organisms, and not only by Cl.welchii. This is described in Section I Part 3. A few experiments were also carried out in an attempt to produce "positive" presumptive tests in MacConkey /

MacConkey broth with organisms commonly found in milk, also with combinations of these organisms. This is described in Section I Part 4. The latter part of the investigation involved an attempt to correlate two other tests with the plate count and presumptive coliform test. These were the Resazurin dye reduction test and the psychrophilic count. The latter was carried out to see if it gave a better indication of the quality of milk because the plate count at 37°C has been criticised on the grounds that most of the bacteria found in milk have optimum growth temperatures at 30°C or below and may multiply at refrigeration temperature, and may be dominant in the flora of milk held at atmospheric temperature (Thomas et al., 1949).

Psychrophilic Organisms in Milk

The term psychrophile has been used to describe a wide variety of bacteria ranging from those growing at 1-2°C to those growing up to 20°C It appears to be a general term for bacteria with optimum temperatures below 20°C (Thomas & Sekhar, 1946). These authors found that all cultures isolated at 3-5°C grew well at 22°C after 2-days' incubation. If low incubation temperatures are used (3-5°C) longer incubation periods are required e.g. 14-21 days.

Milk is often held at refrigeration temperature for some time before delivery, and psychrophiles may multiply rapidly and be present in large numbers, although they are not detected by the 37°C count or dye reduction tests. Bulk milk cooling on farms and bulk collection of milk by refrigerated road tankers is becoming more common in Scotland. Milk may remain for 2 days in a farm tank at a temperature of 4°C before it is collected by the tanker. While this retards the growth of many bacteria, it will not retard the growth of psychrophiles. Thom (1959) has shown that storage of milk at 4°C for 24 hours can result in a twenty-fold increase in the psychrophilic /

psychrophilic count.

These organisms are mostly found in water and belong to the Achromobacter, Flavobacterium, Pseudomonas groups and some are micrococci (Thomas & Sekhar, 1946). Low coliform counts are not necessarily an indication of low psychrophilic counts and high psychrophilic counts may be obtained in the absence of coliforms (Nelson & Baker, 1954). In fact, coliform organisms are known to multiply rapidly in milk held at 2-4°C (Dahlberg, (1946) Fig. 2A, page 20) and Klebsiella and Citrobacter species are quite common in such milk (Thomas, 1958). Thomas et al., (1949) have compared Psychrophilic counts of raw and pasteurised milk and laboratory pasteurised milk. The latter contained no psychrophiles, 75% of farm milks had counts of over 10,000/ml. compared with 37% of commercially pasteurised milks. As the incidence in pasteurised milk is much lower, and since the psychrophiles are virtually absent from laboratory pasteurised milk and from "in line" samples taken from pasteurisation plants the authors suggest that their presence in pasteurised milk is due to contamination from bottles and filters. The organisms are present in water and will probably remain in water residues in the bottles. It is suggested that a psychrophilic count would be a useful index of post-pasteurisation contamination. There are indications that plate counts at the lower temperatures may be useful in examination of bulk-cooled milk (Thom, 1959). There, the temperatures are very low and large numbers of bacteria unable to grow at 37°C may multiply rapidly and be responsible for the development of taints which would remain undetected by the tests at 37°C. Perhaps what is more important is the way in which the count at 27-30°C may rise and fall without there being any comparable variation in the 37°C counts. Miss Thom has shown that two farms with approximately the same 37°C counts for the /

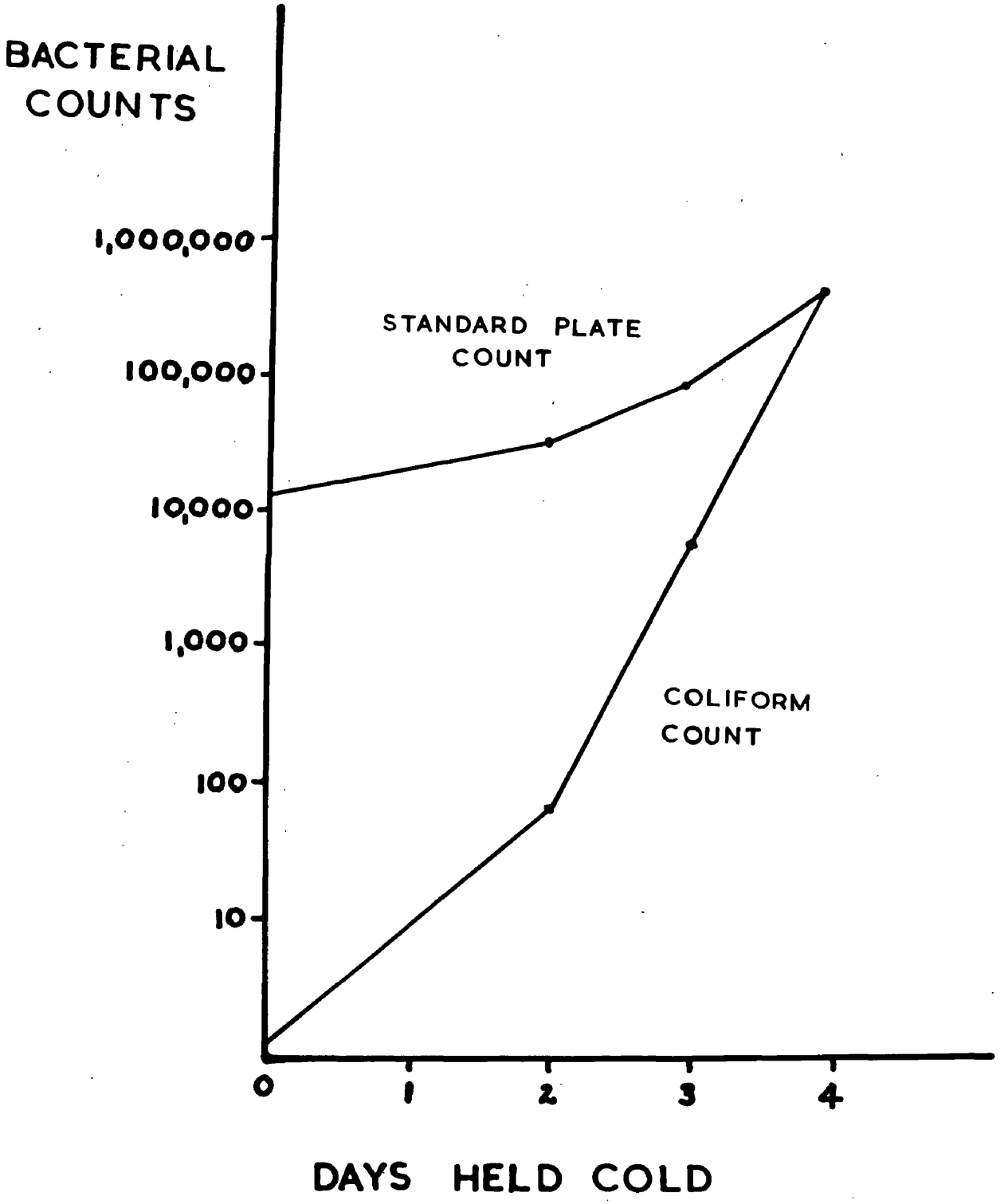


Fig. 2A. Comparison of total counts and coliform population in milk held at 7-10°C for 4 days. (from Dahlberg, 1946).

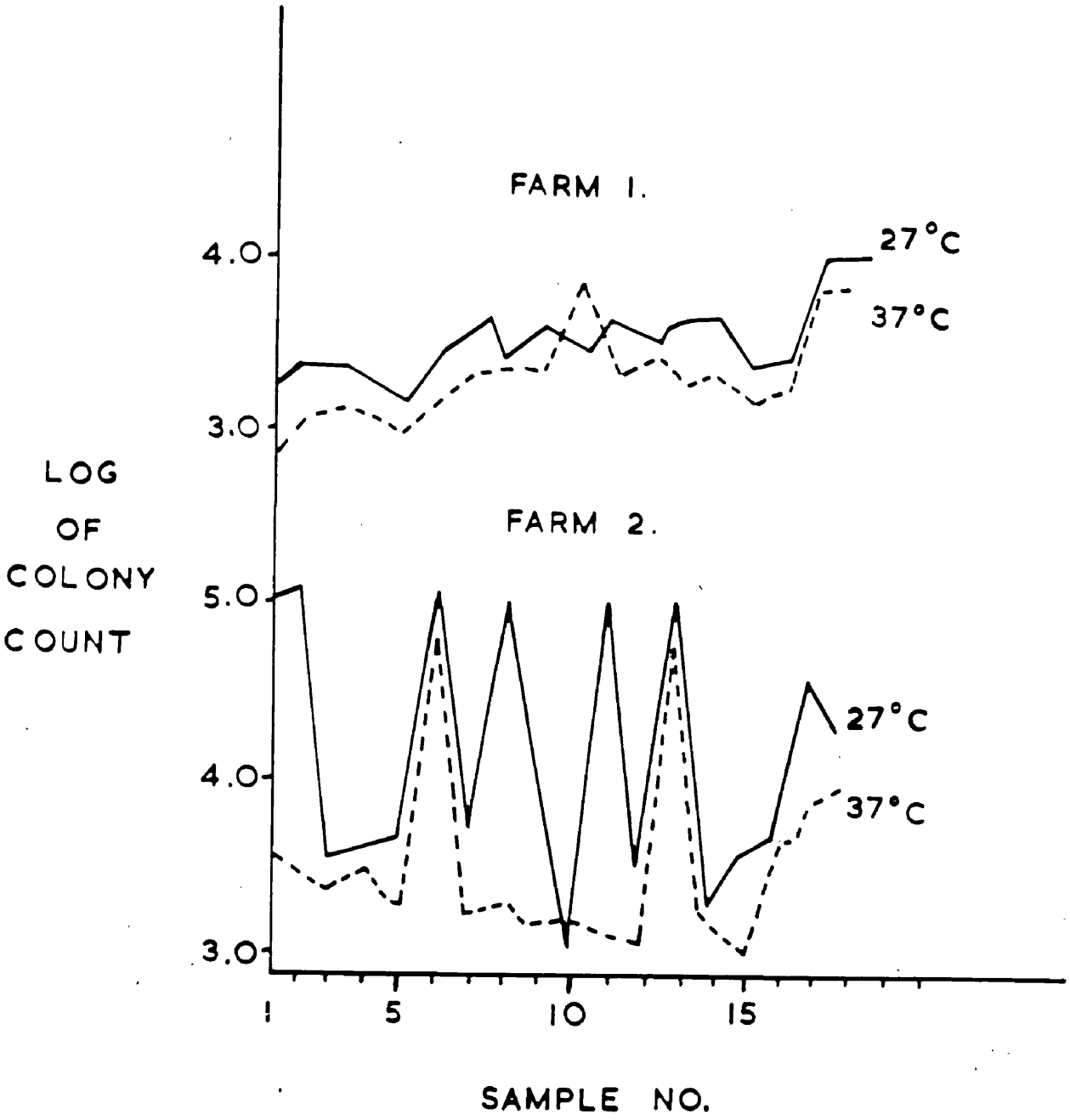


Fig. 2B. Comparison of plate counts at 27°C and at 37°C for two farms (from Thom, 1959).

the same number of samples may differ considerably in their 27°C counts. (Fig. 2B page 21).

At farm 2 the 27°C count shows a series of peaks, many of which are not reflected by the 37°C count. It was found that where there is a 27°C peak, large numbers of Pseudomonas, often in pure culture, and other Gram-negative rods, were present. These organisms will multiply rapidly at bulk-tank temperatures, and it is desirable that tests on such milk should be capable of assessing their numbers.

Smillie (1958), found that 50% of his tanker milks would have failed to pass plate counts at the lower temperatures. Dahlberg (1946), carried out colony counts on pasteurised milk which had been held at various refrigerator temperatures. Parallel counts were made on ordinary agar of total counts. He found that after storage at 45-50°F (7-10°C) for 4 days, coliforms constituted about 5% of the total count in October and during July and August, the coliform count became 88% of the total. There was a much greater relative growth of coliforms as can be seen from Fig. 2A (page 20).

Smillie (1958) suggested that in bulk tanker milks a psychropilic count standard of 10,000/ml. would be a better index of the quality of the milk than the present standard plate count. With these more recent observations in mind, and the knowledge that increasing amounts of milk are being collected by the bulk tanker system, it was decided to determine psychrophilic counts of "Certified" and Pasteurised milk to see how they compared with the standard plate and coliform tests. As most psychrophilic bacteria appear to grow well up to 22°C the low incubation temperature of 4°C was not employed. Counts were incubated at 10-18°C for 3 days and this constituted the "psychrophilic" count. These temperatures may be too close to the minimum temperatures of mesophiles to give adequate separation (Lancet annotation 28th November, 1959).

The /

The Resazurin Test

The Resazurin test, like the methylene blue reduction test, is based on the reduction of a dye to a colourless compound by reducing systems in milk. The main reducing systems are those set up by the metabolism of bacteria (Hobbs, 1939), but others, slightly less active, may be caused for example, by large numbers of leucocytes. In this way the extent to which the dye is decolourised, or the time taken to reach a certain degree of decolourisation has been taken to indicate bacterial activity in milk. Reduction of the dye takes place in two distinct stages. During the first stage resazurin is reduced to resorufin passing through a series of colour changes from blue, lilac, mauve, pink-mauve to pink. The second stage, reduction of pink resorufin to colourless dihydro-resorufin, is reversible and may be catalysed by exposure to oxygen. These colour changes may be matched in the Lovibond comparator and the results reported by the readings for the appropriate changes.

When the test was first brought into use results were not consistent due to variations in the composition of resazurin. This has been overcome by the production of standard resazurin tablets specially prepared for the purpose. The temperature of 37°C will not allow full activity of all the organisms in milk and all apparatus and reagents must be sterile as the dye is very sensitive to both bacterial and non-bacterial reducing systems.

The test has sometimes been applied to measure the keeping quality of milk (Clegg et al., 1949). Results have only been partly successful. Extensive experiments by various workers (Rowlands et al., 1950, Eddison et al., 1951, Rowlands & Hosking, 1951) have shown that the use of a dye-reduction test is not advisable for measurement of the keeping quality of milk. Garvie & Rowlands (1952) have shown that the types of bacteria which were dominant at certain times /

times in samples examined were not always the same. Milk incubated at 37°C (temperature used in resazurin test) showed an increase in staphylococci and streptococci. Incubation at 22°C encouraged mainly milk streptococci. Incubation at 37°C favoured rapid development of types which would grow slowly at 22°C and would therefore not be predominant in milk which was stored under normal conditions of household storage. Hobbs (1939) found that the organisms most active in reducing methylene blue at 37°C were coliforms, Strep. lactis, and Staph aureus.

The results of the Resazurin test are influenced by the temperature at which the milk has been kept before testing. In wintertime bacteria present in milk do not multiply so quickly as in summertime and a temperature-compensated resazurin test has been introduced to allow for this effect and is used in the routine examination of milk under the Scottish Milk Testing Scheme (1955). The incubation time at 37°C is altered according to the atmospheric shade temperature at the time of testing. Even this modification is not satisfactory because many poorly produced milks are not detected in wintertime. A further modification of the test has been suggested (Morgan, 1959). This consists of carrying out the compensated test in the usual way, reading the test, and then incubating the tubes at 20°C till the following day. This modification is for use only during the months October to May and is said to be able to fail milks with counts of 70,000-200,000/ml. Although this test is apparently favoured by Department of Health Authorities and producers because of the simple method involved, I consider that too many factors have to be adjusted before the results of the test are obtained e.g. (1) The dye resazurin itself is not very stable and is reduced by sunlight; (2) other reducing systems in milk e.g. leucocytes will reduce the dye and a period of incubation /

incubation of 24 hours at atmospheric shade temperature is required before testing. This enables the bacteria present to multiply, thereby rendering other reducing systems less effective; (3) the test is not so efficient in winter months for the reasons already stated and further modifications are required. It seems that a test which requires so many modifications cannot be regarded as "simple" and "reliable" when testing milk. If a winter milk has a low bacterial count it will pass the resazurin test yet might fail it in warmer summer months after the period of holding at 24 hours before testing. Experiments in this piece of work have shown that milks passing the resazurin test very often fail both coliform test and plate counts and may contain coliforms of the faecal type I variety. Nilssen (1959), has shown that mastitis milk has considerable reducing properties because leucocytes and the presence of a substrate for xanthine oxidase cause a rapid fall in potential of milk. Methylene blue itself takes part in reducing actions, and it may be that resazurin similarly affects the rate of reduction.

Tetrazolium Salt Media for the Detection of Coliforms

A new type of reduction test, involving the use of tetrazolium salts has been suggested by German workers (Schönberg, 1954, Kraus, 1957). Actively metabolising bacteria reduce triphenyltetrazolium chloride to a reddish-purple formazan. Above certain concentrations, tetrazolium salts are inhibitory to most organisms but coliforms can withstand much higher concentrations than other bacteria.

These workers claim that with an acid (pH. 6.4), 0.22% triphenyltetrazolium chloride broth, coliforms can be detected in a matter of 3-6 hours and emphasise its usefulness for examining milk and water samples and for the detection of pathogenic serotypes of E.coli. Chapman (1951) on the other hand described a solid selective medium /

medium containing triphenyltetrazolium chloride (TTC) for the quick detection of E.coli. This depended on the fact that E.coli although able to grow in high concentrations of TTC, did not reduce it to a red formazan, whereas the other coliforms did. Colonies of E.coli on this medium are greenish-yellow and other coliforms produce dark red colonies.

According to Schönberg (1954) and Kraus (1957), E.coli reduces TTC and this is supported by a description of TTC staining of E.coli (Eidus et al., 1959). These facts seemed to promise an interesting method for the quick detection of coliforms in milk if the various contradictions could be settled. I therefore carried out some experiments to find the limiting concentration of TTC required to allow growth of coliforms and yet inhibit most other organisms normally found in milk with the possibility of adapting a TTC broth to replace the conventional 48-hour coliform test in MacConkey medium. The results of these experiments are described in Section I Part 5.

N.B. Due to the constant changing of nomenclature and classification of the Enterobacteriaceae, an explanation of the names used in this work is necessary. Coliforms are referred to by the names suggested by the Coli-aerogenes (1956) Sub-committee. When discussing the work of other authors, or quoting the results of other workers, the nomenclature employed will be that of the original papers.

Materials and Methods /

Materials and Methods

Media

1. The medium employed for the presumptive coliform test was MacConkey's single strength, lactose, bile salt broth made from Oxoid granules and of the following composition.

Bacteriological peptone	(Oxoid)	20 gm.
Bile Salts	(")	5 "
Lactose	(")	10 "
Sodium chloride	(")	5 "
Neutral red indicator		0.05 "

pH approx. 7.4

The medium was tubed in 6 ml. amounts in 6" x $\frac{5}{8}$" test tubes with small Durham tubes to collect gas, and sterilised at 10 lb./sq.inch for 20 min. Reactions were read as positive if the medium was acid and the Durham tube contained even a small volume of gas. (In the standard method the test is positive only if at least one-third of the Durham tube contains gas).

2. Positive tubes were plated on to MacConkey agar prepared from Oxoid granules. The composition of this was as for MacConkey's broth with the addition of:-

Agar-agar (Oxoid) 12 gm.

pH approximately 7.4. Sterilised at 10 lb./sq.inch for 20 min.

3. Dilutions of milk were prepared in quarter-strength Ringer's solution prepared from Oxoid tablets and having the following composition:-

Sodium chloride	9.0 parts
Potassium chloride	0.42 "
Calcium chloride	0.48 "
Sodium bicarbonate	0.20 "

Solution was sterilised at 15 lb./sq.inch for 20 minutes.

4. /

4. The medium employed for milk-plate counts was Oxoid milk agar prepared from granules and of the following composition:-

Bacteriological yeast extract	(Oxoid)	3 gm.
Bacteriological peptone	(")	5 "
Agar-Agar	(")	15 "

Milk equivalent to 10 ml. fresh milk.

pH approx. 7.2

Sterilised by 10 lb./sq.inch for 20 minutes.

The above media are all made to a formula similar to the official one described in the Ministry of Health publication Memo 139/Foods 1937 for examination of milk.

5. Inocula for biochemical reaction tests were obtained by growing colonies taken from MacConkey agar in peptone water medium of the following composition:-

Peptone (Oxoid)	2%
Sodium chloride	0.5%
Distilled water	
pH 7-7.2	

Sterilised at 5 lb./sq.inch for 30 minutes.

6. Fermentation of sugars was tested in the basal peptone water medium described above with the addition of 1% Carbohydrate for lactose, glucose, sucrose, maltose and mannitol, and 0.5% for dulcitol. The indicator was Andrade's indicator 1% Sterilised by steaming for 20 minutes on 3 successive days. Other biochemical tests included a tube of litmus milk. A tube of peptone water was employed to test for indole production.

The methyl-red and Voges-Proskauer reactions were tested in glucose /

glucose phosphate broth of the following composition:-

Potassium dihydrogen phosphate	0.5%
Peptone (Oxoid)	0.5%
Glucose	1%
Distilled water	

pH 7.5. Sterilised at 5 lb./sq.inch for 15 minutes.

Ability to grow in an inorganic carbon medium where the sole source of nitrogen is also inorganic was tested in ammonium citrate medium of the following composition:-

Sodium chloride	5.0 gm.
Magnesium sulphate	0.2 "
Ammonium dihydrogen phosphate	1.0 "
Dipotassium hydrogen phosphate (anhydrous)	1.0 "
Distilled water	1000 ml.

pH 6.8. Sterilised by steaming for 20 minutes on three consecutive days.

Isolation of Cl. welchii - Media employed

1. Litmus milk for preliminary isolation had the following formula:-

Dried skimmed milk (Oxoid)	100 gm.
Litmus	q.s.
pH approximately 6.8	

The medium was distributed in 6" x $\frac{5}{8}$" test tubes and sterilised by steaming at 100°C for 30 minutes on 3 successive days.

A variation on the above medium sometimes employed a strip of iron metal in the tube.

2. Robertson's Meat Medium for further culture was prepared from 'Oxoid' /

'Oxoid' tablets and had the following composition:-

Bacteriological peptone ('Oxoid')	10 gm.
'Lab-lemco'	10 "
Sodium chloride	5 "
Neutral heart issue	30 "

pH approx. 7.6. Sterilised by autoclaving at 10 lb./sq.inch for 20 min.

3. Blood agar plates were employed to obtain colonies for pure culture. These were prepared by adding 7% sterile "Wellcome" normal horse blood (oxalated) to melted, cooled, (to 56°C) "Lab-lemco" agar ('Oxoid').

All media employed for the isolation of Cl. welchii were incubated anaerobically in a McIntosh & Fildes anaerobic jar with the exception of the litmus milks containing iron strips. These were incubated aerobically.

Methods

1. Presumptive Coliform Test

Bottles of milk were inverted rapidly about 10 times to mix the contents and approximately 20 ml. withdrawn with a sterile pipette and placed in a sterile universal container. This was the milk sample examined. The presumptive test was carried out by making serial tenfold dilutions (1 in ten, 1 in a hundred, 1 in a thousand) of milk in 9 ml. amounts of sterile quarter-strength Ringer solution. Starting with the 1 in a thousand dilution, 1 ml. of dilution was added to each of three tubes of MacConkey broth. Using the same pipette 1 ml. of the 1 in a hundred and 1 ml. of the 1 in ten dilutions were added to 3 tubes of medium, ending with the addition of 1 ml. of the original milk sample to the last three tubes. Tubes were incubated at 37°C and read after 48 hr. This time and temperature of incubation was chosen as it /

it is the one specified at present in the Milk (Special Designations) (Scotland) Order 1951.

2. Plate Count

Using the same dilutions of milk as for the presumptive test, 1 ml. of 1 in a thousand dilution was transferred to each of two sterile Petri dishes, and 1 ml. of 1 in a hundred dilution to each of four Petri dishes. Approximately 15 ml. of melted, cooled, milk agar was then poured into the dish and thoroughly mixed with the dilution in the standard way. Plates were incubated at 37°C and counted after 48 hours. For the psychrophilic plate counts, incubation was at 10-15°C for three days. The choice of 1 in a hundred and 1 in a thousand dilutions and the absence of 1 in ten dilution for the plate count was made to avoid wide discrepancies which seem to appear most often in 1 in a hundred dilutions (Malcolm, 1932). Hence 4 plates were poured for this dilution. The plates were incubated at 37°C and counted after 48 hours according to the standard laid down in the Milk (Special Designations) (Scotland) Order 1951. The incubation temperature of 37°C has recently been criticised (McKenzie, 1952) as many of the organisms normally found in milk have optimum temperatures lower than this. It is suggested that 30-32°C for 3 days is a more suitable temperature. Chalmers (1955) however, suggested that although the counts at these lower temperatures may be as much as 50% greater than those at 37°C, the ratio of the counts varies depending on the flora of the milk. It is doubtful whether there would be any marked advantage in changing the temperature for routine examination as this is not a measure of the exact number of organisms present, but a means of comparing the degree of contamination of milk supplies.

3. /

3. Differentiation of Coliforms

The positive tubes of the highest or second highest milk dilution were plated on to MacConkey agar and incubated for 18-24 hours at 37°C. Lactose-fermenting colonies were picked up into peptone water, incubated for 6 hours, and used to inoculate media for biochemical reactions. Gram-stained films were prepared from colonies to see whether Gram-negative rods were present. Biochemical media were inoculated with one drop of peptone-water culture with the exception of the citrate medium which was inoculated with a straight nichrome wire. A control tube of meat-extract broth was inoculated immediately after this without sterilising the wire to ensure that negative reactions were not due to faulty inoculation. Biochemical reactions were incubated at 37°C for 24-48 hours. Positive sugar reactions were shown by the indicator's turning red in presence of acid and by collection of gas in the Durham tube. Presence of indole in peptone water was detected by adding a few drops of Ehrlich's rosindole reagent - a positive reaction giving a pink colour. The methyl-red test was carried out by adding two drops of methyl-red indicator to a tube of glucose-phosphate broth. A positive reaction was red, negative yellow. Test for presence of acetyl-methyl carbinol was made by adding 0.6 ml. of V.P. reagent 1 (5% alcoholic α-naphthol and 0.2 ml. of V.P. reagent 2 (40% KOH) to the other tube of glucose - PO_4 medium and shaking. Reaction was read after 1 hr., a positive reaction being red. Growth in citrate medium was indicated by turbidity. The latter 4 tests will be referred to as 'I.M.V.I.C.' reactions.

4. Isolation of Cl. welchii

Approximately 3 ml. of milk sample was placed in a 4" x $\frac{1}{2}$" test tube and held in a water bath at 80°C for 10 min. It was then transferred /

transferred to a tube of litmus milk and incubated at 37°C in the anaerobic jar, or to a tube of iron-litmus milk and incubated aerobically for 24 hours. A "stormy clot" was taken to mean presence of Cl. welchii but was further investigated by trying to isolate the organism in Robertson's meat medium and on blood agar plates incubated anaerobically. Confirmation was by Nagler reaction and sugar fermentations. These techniques are described in the section dealing with this organism.

5. Staphylococcus aureus

Materials and methods are described in the section on Staph.aureus as this was a separate piece of work which was completed some time before the work on coliforms.

6. Differentiation of 'Intermediate' coliforms

When some coliforms with the 'I.M.V.I.C.' reaction of ++++ type were isolated, it was decided to examine them more closely to see whether they might indeed be Klebsiella or Cloaca types. The method employed was that of Møller (1954) for determining amino-acid decarboxylase systems. The amino acids used were (L+) lysine, (L+) arginine, (L+) ornithine and (L+) glutamic acid in 1% concentration in basal medium of the following composition:-

Peptone (Eupeptone)		5 gm.
Beef extract ('Lab-lemco') ('Oxoid')		5 "
Brom-cresol purple (1:500)		5 ml.
Cresol red (1:500)		2.5 "
Pyridoxal		5 mg.
Glucose		0.5 gm.
Water	ad	1000 ml.
pH adjusted to 6.0		

Approximately 1.5 ml. of basal medium containing the above amino acids was placed in 4" x ½" test tubes and steamed for 20 min. on /

on 3 successive days. A thin layer of sterile liquid paraffin was placed over the medium. Tubes were inoculated from agar-slope cultures with a straight wire. All tubes were incubated at 37°C including a control tube with no amino acid. A tube of glutamic acid medium was also incubated at 25°C .

Tubes were read daily, change of colour from yellow to violet indicated a positive reaction.

The glutamic acid medium received special treatment after 24 hr. 0.2 ml. of 0.25N HCl was added to both tubes and shaken. Further incubation for 48 hr. for 37°C tube and 5 days for 25°C tubes was followed by addition of 0.25 N NaOH and final colour changes read.

7. Resazurin Test

10 ml. of milk was transferred to a sterile 6" x $\frac{5}{8}$" test tube closed by a rubber stopper. To this was added 1 ml. of a standard resazurin solution prepared from standard resazurin tablets supplied by B.D.H. The solution was prepared by dissolving 1 tablet in 50 ml. of sterile, cold, glass-distilled water. The solution was prepared freshly for each test. The tube containing dye and milk was inverted to mix the contents and incubated in a water bath at 37°C. Tubes were examined after 1 hour and after 6 hours. Colour was measured in the Lovibond comparator fitted with a special resazurin disc. Quality of the milk was judged according to the following scale /

scale

Colour after 1 hour at 37°C	Disc number	Quality of milk
Blue	6)	
Lilac	5)	Satisfactory
Mauve	4)	
Pink-Mauve	3)	
Mauve-pink	2)	Fair
Pink	1)	
Colourless	0	Poor

There have been several modifications of the Resazurin test including the Temperature-Compensated Resazurin test used for the routine examination of milk under the Scottish Milk Testing Scheme. Because the age of the samples was not known, nor the temperatures at which they had been stored since production, it was impossible to carry out this modification on the samples tested.

However samples were incubated for at least 5 hours before being discarded. The maximum time for incubation in the Temperature-Compensated test is 2 hours. Milk giving a disc reading of $3\frac{1}{2}$ or less from April to October, or $4\frac{1}{2}$ or less from November to March, was considered to have failed the test.

The standards with which the milks examined were compared are those set down in the Fourth Schedule of the Milk (Special Designations) (Scotland) Order 1951 and are reproduced below:-

"Certified" Milk

(a) Must not contain more than 30,000 bacteria per ml.

(b) " " " coliform bacteria in a 0.1 ml.

Pasteurised Milk

(a) Must not contain coliform bacteria in a 0.01 ml.

In /

In many cases 'positive' MacConkey tubes were obtained in dilutions of milk which would be ignored by the standard test level e.g. 1 ml. for "Certified" or 0.1 ml. for Pasteurised. These were also examined for the presence of coliform bacteria and recorded as 'positive'. The two results however are reported separately as will be seen in the following tables. Tubes were read as 'positive' even if the gas volume in the Durham tube was small. Very often 'false' positive tests had small gas volumes (Plate 1 page 131).

Plan of Experiments

An attempt was made to obtain a reasonable selection of milk samples of three grades - "Certified", Tuberculin tested and Pasteurised, throughout the year and to compare the number of positive coliform tests at different seasons. The organisms responsible for the positive reactions were isolated to see whether they were indeed coliforms and of what type they were. Attempts were made to identify organisms responsible for 'false' positive tests. Plate counts were carried out on the various samples to compare them with the presumptive test. Due to the difficulty in obtaining unpasteurised tuberculin-tested milk, and to the fact that it is fairly similar to "Certified" milk in origin, it was decided to compare only "Certified" and Pasteurised milks in later experiments.

Samples were also examined for Cl. welchii and during 1956-57 (See Section I Part 3) and for Staph. aureus (see Section II). These surveys were carried out during 1956-57 and 1957-58. During 1958-59 the plan of experiments was altered slightly. Detection of Cl. welchii was omitted and Resazurin tests and plate counts at 10-18°C were carried out in an attempt to relate these to the presumptive coliform test. (See Section III).

RESULTS

Section I

Survey of Consumer Milk for the years 1956-57 and 1957-58 at different seasons of the year.

Part I. Presumptive Coliform Test

(a) Pasteurised Milk

Table No. 1 (page 38) shows the incidence of positive presumptive tests (as judged by standard) and positive tests in any other dilutions for the years 1956-58.

Table No. 2 (page 39) shows the types of coliform organisms found in positive tests.

From the figures shown in these tables it can be seen that a much larger number of samples fail during summer than in winter, and that the failures in spring are very variable. This may be due to the indefinite limits of "spring" (taken here from the middle of February to the end of May) and to the variable temperatures encountered during this period. Although theoretically, pasteurised milk should contain no coliforms, these organisms are often present due to post-pasteurisation contamination (e.g. dirty bottles) and it is expected that there would be a higher incidence of positive tests in summer when atmospheric temperatures will favour multiplication of bacteria. It has been shown (Mattick et al., 1939) that if the minimum strength of alkali is not maintained throughout the whole working time of bottle washing machines, glass bottles may be heavily contaminated. Bottles are also liable to heavy contamination by rinse water in later stages. Chalmers and Sampson (1958) report an incidence of 0.6% positives in winter and 5.8% positives in summer for 1957. These figures are much lower than the ones obtained in the present /

Table 1. Incidence of Positive Presumptive Tests in Pasteurised Milk for 1956-58

SEASON	1956-57					1957-58				
	Number Samples Examined	Number Positive by Standard	% Positive by Standard	Number Positive in any Dilution	% Positive in any Dilution	Number Samples Examined	Number Positive by Standard	% Positive by Standard	Number Positive in any Dilution	% Positive in any Dilution
WINTER	27	0	0	5	18.5	27	2	7.4	7	25.9
SPRING	19	5	26.3	10	52.6	23	0	0	2	8.7
SUMMER	22	6	27	10	45.4	19	8	42	10	52.6

N.B. WINTER - October, November, December, January, February.

SPRING - March, April, May.

SUMMER - June, July, August, September.

Table 2. Types of coliforms isolated from positive tests in Pasteurised Milk 1956-58.

SEASON	1956-57					1957-58				
	Coli	Klebsiella	Intermediate	Paracolon	Other Organisms	Coli	Klebsiella	Intermediate	Paracolon	Other Organisms
WINTER	-	2	-	-	3	1	-	4	-	2
SPRING	1	6	3	-	1	1	-	-	-	1
SUMMER	2	2	5	-	1	0	8	2	-	-

present survey but it must be remembered that the number of samples I have examined is much smaller, and that the milk may have been standing at a fairly high temperature for a day or more before purchase. My figures for samples failing at any dilution at all give an even higher failure. Plotting these figures on a graph, it can be seen that the percentages failing at standard dilutions and those failing at any dilution give two parallel curves. (Figs. 3A page 41 and 3B page 42). This shows that seasonal variations in temperature neither affect the technique of the test nor the incidence of "false" positives.

If the positive tests are examined for the types of coliforms causing the reaction (Table 2 page 39) it can be seen that E.coli is not common in Pasteurised milk - only 3 of 137 tests for the two years yielded this organism and two of these were during summer months.

The organisms isolated most often were K. aerogenes and Intermediate coliforms with no significant predominance of either at any season of the year.

Since coliforms are most likely to gain entrance to pasteurised milk by post-pasteurisation contamination, it is not surprising that the types found most frequently should be those which can survive in milk and water residues of improperly washed bottles and it is thought that organisms of the Klebsiella-Intermediate groups are able to survive longer than E.coli (Bardsley, 1948, Thomas, 1955.) It is possible, however, that the coliforms isolated from pasteurised milk are heat-resistant organisms surviving the temperatures of pasteurisation. Crossley (1946) believes that very few coliforms are heat resistant and that heat-resistant strains gain entrance to the milk from inefficiently sterilised equipment where a population of fairly heat-resistant organisms has been selected. Elliker /

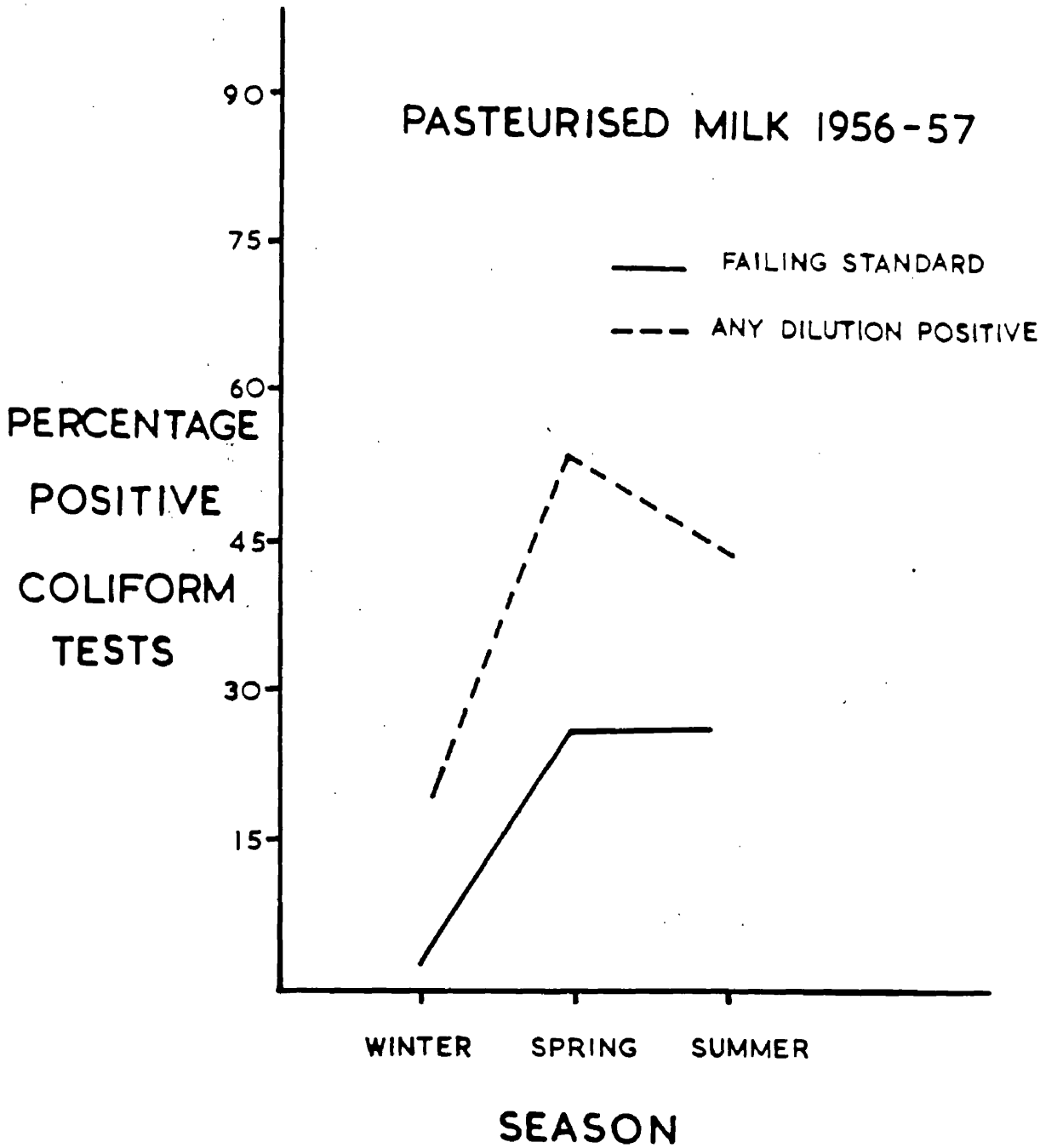


Fig. 3A. Comparison of % failures in presumptive coliform test for pasteurised milk at standard and any other dilutions.

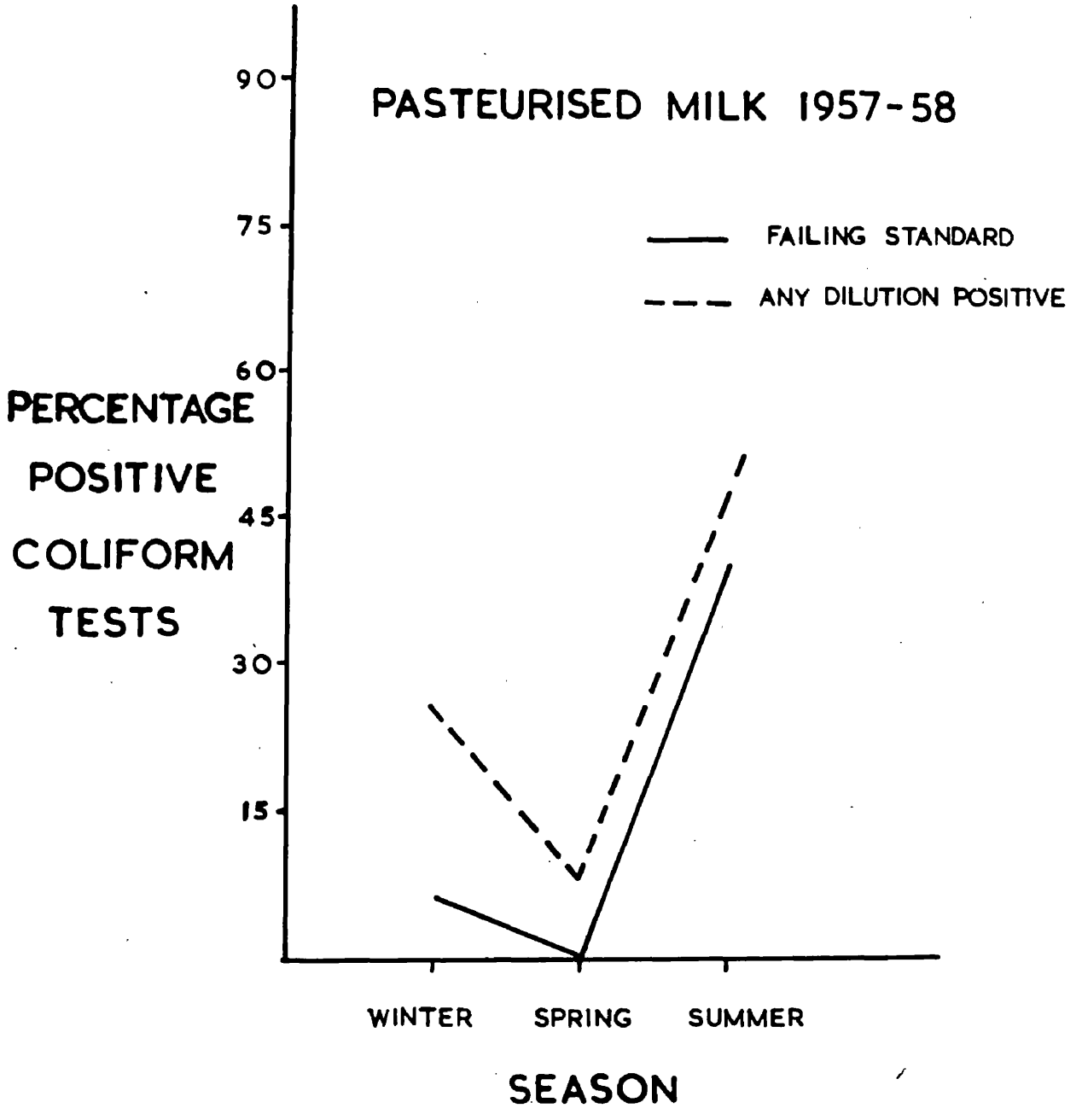


Fig. 3B. Comparison of % failures in presumptive coliform test for pasteurised milk at standard, and any other dilutions.

Elliker & Frazier (1938) have found that the temperature of previous growth prior to heat treatment may affect the heat resistance of E.coli in milk. They suggest that the temperature at which milk is held previous to pasteurisation may affect the efficiency of the process. Of the 137 samples examined over the two years, 8 (5.8%) were caused by organisms other than coliforms. None of these false positives occurred in dilutions which would have failed the milk under the statutory test. The organisms isolated from these "positive" tubes were as follows:-

1. Lactobacillus sp.
2. Lactobacillus sp.
3. Lactobacillus sp. in conjunction with a Gram-negative rod. This organism had polar flagella and fermented a wide range of sugars with the production of acid only. Possibly a Pseudomonas sp.
4. An organism tentatively identified as an Alkaligenes sp.
5. A faecal Streptococcus and an unidentified Gram-positive cocco-bacillus which failed to give any biochemical reactions.
6. A Bacillus sp.
7. An unidentified Gram-positive cocco-bacillus.
8. Clostridium welchii and a Pseudomonas sp. (Plate 2A, Page 132).

In several of these cases, in spite of repeated culturing under both aerobic and anaerobic conditions, only one organism e.g. a Bacillus sp. was isolated. Many of the organisms isolated either did not ferment the various carbohydrates tested or produced only acid fermentation. It is difficult to see how the gas in the positive tube could have been produced. There may be several explanations for this viz.:-

(1) If the coliform organisms were present in very small numbers, they might become quickly overgrown by lactic-acid streptococci and other /

other organisms normally present in milk. These organisms produce large amounts of lactic acid and it is known that there is competition between coliforms and streptococci in milk (Hall, 1957). Initial fermentation begun by coliforms may be arrested by the low pH produced by streptococci, resulting in death of the coliforms. Thus when 48 hours later, the test is read and the positive tubes subcultured, no coliform organisms can be detected.

(2) There may be some antibacterial substance present in the milk, inhibitory to coliform organisms, giving results similar to (1) (Morris, 1945; Morris and Edwards, 1950). This is more likely to happen in raw milk than in pasteurised milk (Morris, undated publication). The substance inhibitory to certain strains of coliforms is completely destroyed by heating to 53°C for half an hour. Jones & Simms (1930) reported an antibacterial substance in fresh milk which was active against streptococci. They named it lactenin and it was destroyed by boiling but it survived temperatures of pasteurisation. Wilson and Rosenblum (1952 A, 1952 B) suggest that lactenin is an enzyme because it is reversibly inactivated by exclusion of oxygen and by sulphur-containing reducing agents *e.g.* cysteine. Lactenin is now known to consist of two fractions L_1 and L_2 which act synergistically, each being of low potency in the absence of the other (Auclair & Hirsch, 1953; Auclair & Berridge, 1953). L_1 is destroyed by temperatures of pasteurisation. Since lactenin inhibits lactic-acid streptococci it may remove the antagonistic effect of these organisms for coliforms. This effect will be greater in raw milk than in pasteurised milk. Auclair (1954) has found that there is also a substance in raw milk which antagonises lactenin. If there is sufficient lactenin activity left in milk after pasteurisation, it may improve its keeping quality.

(3) Many of these false positives appear in tubes which have received

1 ml. of undiluted milk, and not in any others. Very often the gas volume in the Durham tube was small. It occurred to me that the presence of 1 ml. of undiluted milk in MacConkey broth might diminish the selective effect of the medium by enriching it, and thereby overcoming the inhibitory effect of bile salt. Very often the cream layer rose to the surface leaving a fatty scum which might create anaerobic conditions in the tube. Some experiments were carried out later to study the effects of the addition of milk to MacConkey broth on the growth of organisms isolated from false-positive tubes.

(b) "Certified" Milk

Table No. 3 (page 46) shows the incidence of positive presumptive tests (as judged by standard) and positive tests in any other dilutions for the year 1956-58.

Table No. 4 (page 47) shows the types of coliforms isolated from positive tests. From these tables it can be seen that, as for pasteurised milk, the failures are higher in summer than in winter or spring. With "Certified" milk approximately half the samples examined fail at all seasons of the year and in summer the failures are exceptionally high (e.g. 78.2% 85%). The Department of Health for Scotland published results of bacteriological tests for 1956-57 and obtained an overall fail of 22% for "Certified" milk. This is slightly less than an overall fail of 36% found in this work. If however one takes those failing at any dilution a result of 60% is obtained. "Certified" milk is bottled on the farm and is not heat-treated. Although it must be produced under very clean conditions, there are ample opportunities for bacterial contamination if there is any carelessness on the producer's part. Greater contamination will be exaggerated by high summer temperatures. Plotting these figures on a graph, the same picture of two parallel curves is obtained /

Table 3. Incidence of Positive Presumptive Tests in "Certified" Milk for 1956-58

SEASON	1956-57					1957-58				
	Number Samples Examined	Number Positive by Standard	% Positive by Standard	Number Positive in any Dilution	% Positive in any Dilution	Number Samples Examined	Number Positive by Standard	% Positive by Standard	Number Positive in any Dilution	% Positive in any Dilution
WINTER	24	7	29	14	58.3	27	10	37	18	66.6
SPRING	22	6	27	9	40.9	19	6	31.5	10	52.6
SUMMER	20	8	40	17	85	23	18	78.2	21	91.3

Table 4. Types of coliforms isolated from positive tests in "Certified Milk" 1956-58.

SEASON	1956-57					1957-58				
	Coli	Klebsiella	Intermediate	Paracolon	Other Organisms	Coli	Klebsiella	Intermediate	Paracolon	Other Organisms
WINTER	10	1	3	-	-	7	-	11	-	-
SPRING	4	-	5	-	-	8	-	2	-	-
SUMMER	10	-	5	2	-	14	1	5	-	1

obtained as for pasteurised milk. (Figs. 4A page 49 4B page 50). Results with "Certified" milk appear to be more constant than with pasteurised milk. There is a steep drop in positive tests in spring for both years.

The types of coliforms responsible for positive tests differ from those in pasteurised milk. In "Certified" milk E.coli is the type most frequently isolated at all seasons of the year (53 of 135 samples had E.coli). The next predominating type is the Intermediate coliform. K.aerogenes was isolated on only two occasions. With pasteurised milk, however, K.aerogenes was quite a frequent contaminant. There may be various explanations for this viz.:-

(1) K.aerogenes is a saprophytic coliform and will survive in milk residues in improperly washed bottles, utensils, and pasteurising plants, whereas E.coli will tend to die out.

(2) K.aerogenes has a lower optimun growth temperature (30°C) than E.coli. The presumptive test is incubated at 37°C and therefore K.aerogenes although present may not always grow. This would apply to both "Certified" and pasteurised milk but in "Certified" milk where possibly larger numbers of E.coli are present, these organisms will be able to multiply rapidly at 37°C and overgrow any K.aerogenes present.

(3).

K.aerogenes may be more heat-resistant than E.coli or the strains of K.aerogenes surviving in pasteurising plants and sterilised milking utensils may be gradually selected to give fairly heat-resistant organisms.

I did not find a greater number of E.coli in positive tests in winter than in summer, as found by Malcolm (1933). Of the 135 samples examined only 1 (0.7%) was caused by organisms other than coliforms. /

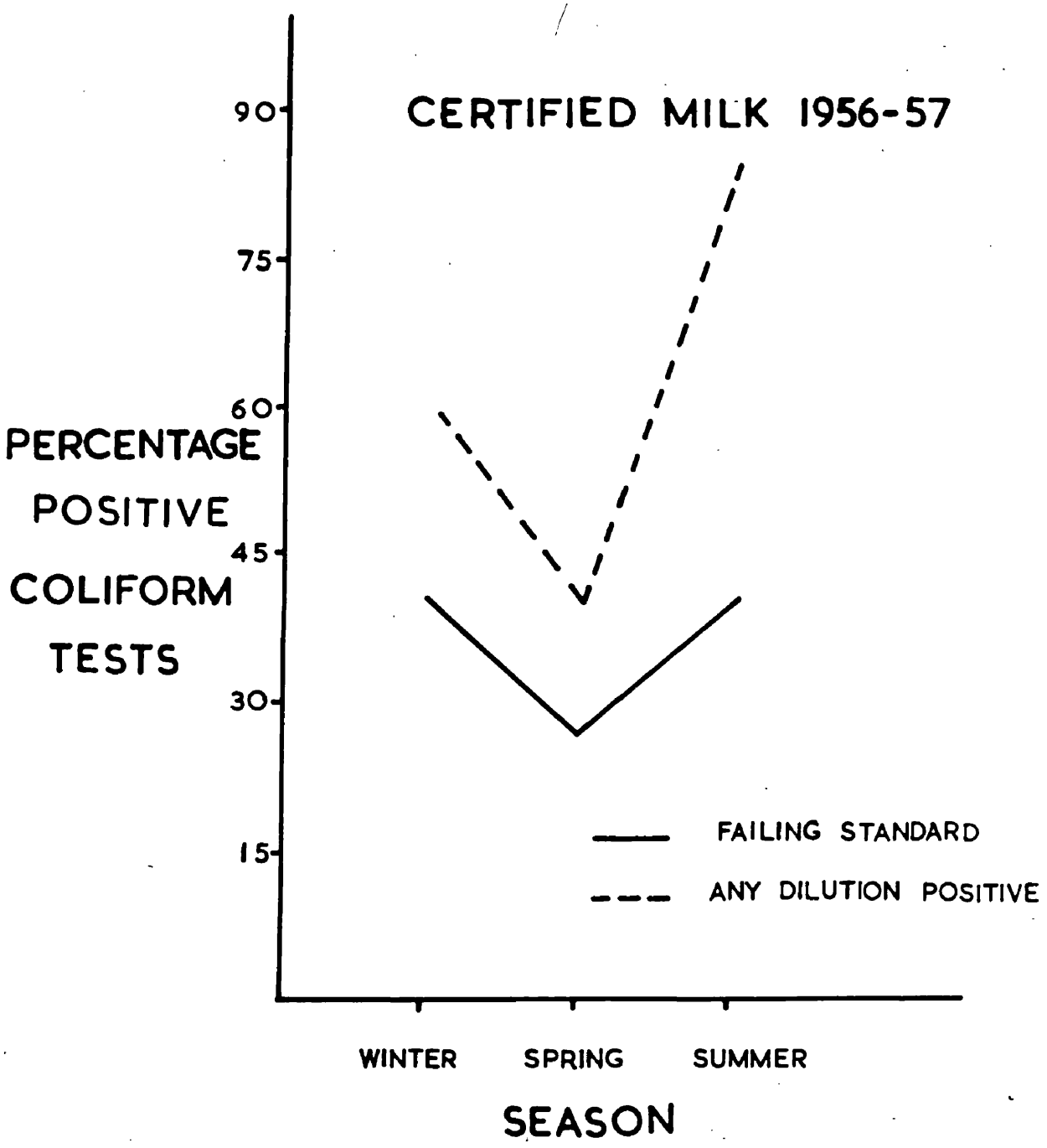


Fig. 4A. Comparison of % failures in presumptive coliform test for "Certified" milk at standard, and any other dilutions.

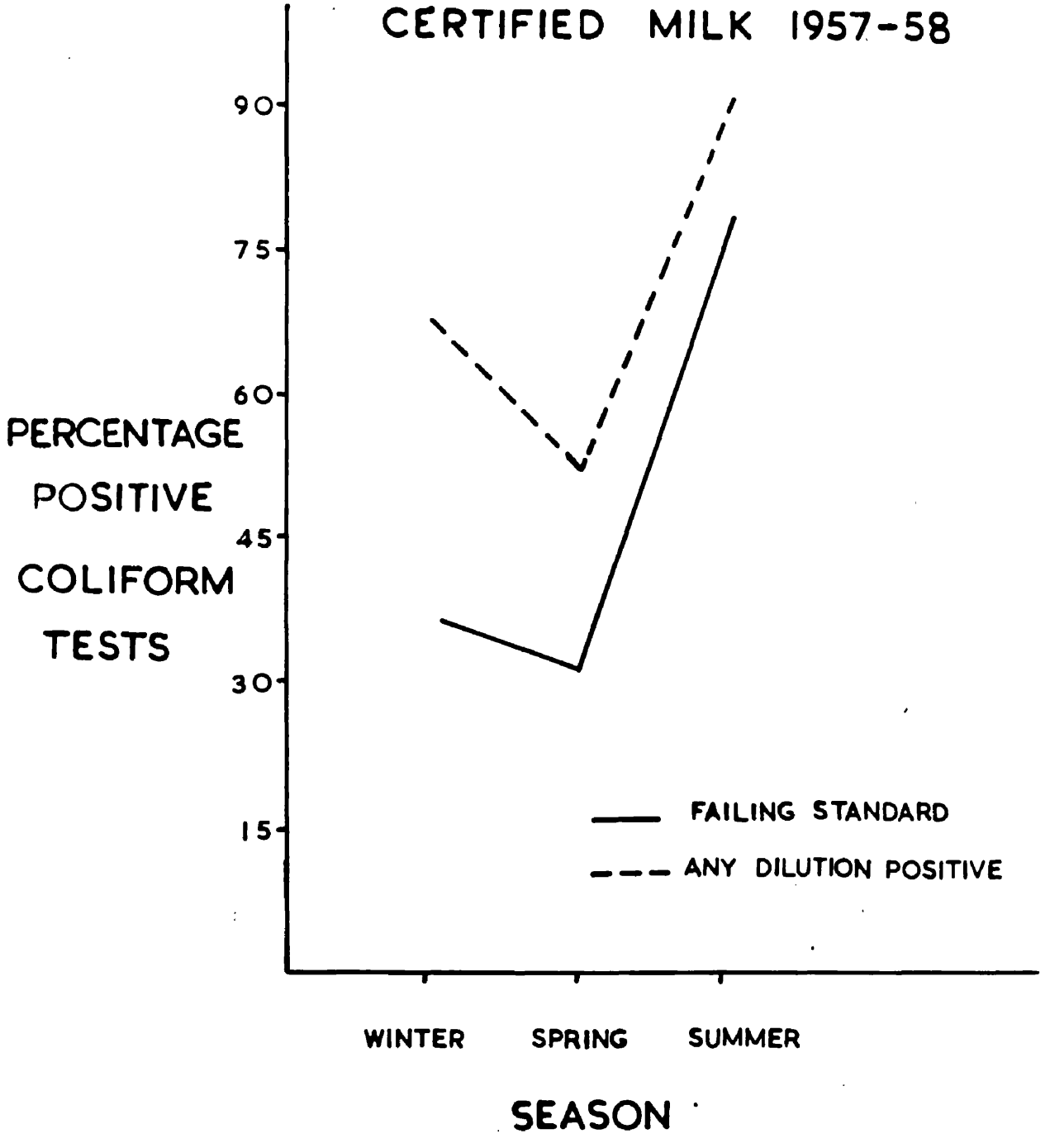


Fig. 4B. Comparison of % failures in presumptive coliform test for "Certified" milk at standard, and any other dilutions.

coliforms. The sample was positive in dilutions which would have failed the test and the organisms responsible were a faecal Streptococcus and a late lactose - fermenting Gram-negative rod. The Gram-negative rod gave good acid and gas production in glucose, mannitol and maltose. Either culture alone did not give a positive MacConkey reaction but if they were added to MacConkey broth together a positive reaction was obtained.

I would suggest that here the false positive has been produced by the synergistic action of the two organisms viz.

lactose $\xrightarrow{\text{streptococcus}}$ galactose + glucose

glucose $\xrightarrow{\text{Gram-negative rod}}$ acid + gas

The streptococcus splits lactose to give glucose which the second organism will split to give acid and gas.

A few experiments were done later to see if this type of reaction might result from two organisms with these properties.

Comparing the false positives with those for pasteurised milk it is at once apparent that they are much more common in pasteurised milk. This seems unusual as "Certified" milk would be expected to have a much wider and varied flora than pasteurised milk. Chalmers (1955) however states that false positives may arise in pasteurised milk, due to anaerobes, and that milk pasteurised by the Holder method tends to give false positives more frequently than when pasteurised by the High Temperature, Short Time method. It is possible that pasteurisation makes the milk population more selective e.g. aerobic and anaerobic spore formers, and these organisms are known to cause false positives (Davis, 1951).

Part 2 Plate Count at 37°C

a) "Certified" Milk

Table No. 5 (page 52) shows the number of milk samples failing the standard plate count at different seasons of the year for the years 1956-58.

Table No. 5 Plate-count failures in "Certified" Milk for 1956-58

	1956-57			1957-58		
Season	Number of samples examined	Number failing	% failing	Number of samples examined	Number failing	% failing
Winter	24	1	4	27	3	11.1
Spring	22	3	13.6	19	1	5.2
Summer	20	6	30	23	16	69.5

Table 6 - Comparison of Failures on the Plate Count at 37°C and Presumptive Coliform Test for "Certified" Milk during 1956-58

	1956-57		1957-58	
Season	Number of samples failing plate count	Number of samples failing both tests	Number of samples failing plate count	Number of samples failing both tests
Winter	1	1	3	2
Spring	3	3	1	1
Summer	6	6	16	15

As would be expected, there is a higher proportion of failures in summer than in winter or spring. The overall percentage failure for 1956-57 is 15% which is much greater than the 5% recorded by the Department of Health for Scotland for that year. This difference may be due to the small number of samples which I examined. However many of the samples which failed the plate count yielded so many colonies that the plates were obviously uncountable. These samples were heavily contaminated, or had been stored for some time at a fairly high temperature, allowing bacterial multiplication to take place.

If the plate counts are compared with the coliform test, it can be seen that in every case except two, failure on the plate count was accompanied by failure in the coliform test (Table 6, page 53).

One of the samples not failing the coliform test was completely negative and the other had an Intermediate coliform present but not in the dilutions which would have failed it by the standard.

From these results it would appear that a high count is usually associated with coliform failure. Although there are no published references available, milk bacteriologists frequently observe milks which have low counts but which fail the coliform test even in high dilutions. In weighing this problem, it is known that coliforms and other organisms may compete (Parmala, 1956). Discrepancies between plate counts and coliform tests have given rise to complaints in the farming world where samples which would have passed the plate count, have been failed by the coliform test. This argument is often put forward in favour of abandoning the coliform test.

I think that the explanation of a discrepancy between these /

these two tests is much simpler than one would believe. It is known that the coliform test for water will detect the presence of one organism in 100 ml. of water and it is assumed that only one organism need be present to produce acid and gas in MacConkey broth. The same is probably true for milk.

If one organism is present in, say, the 1 in a hundred dilution tube, it will give a positive coliform test. The same organism on the 1 in a hundred dilution plate will give rise to only one colony which will not appreciably increase the plate count. The single organism in the MacConkey tube however, may multiply rapidly giving rise to a sizeable population and produce much acid and gas. There is also the possibility that the milk contains either penicillin or a bactericidal substance, e.g. lactenin, which will inhibit the normal milk flora of streptococci and staphylococci, lower the plate count, and yet not affect the coliform test. It is known that penicillin in milk prolongs the reduction time in the Methylene Blue Test, inhibits the growth of some contaminating organisms, and interferes with the reliability of bacteriological milk tests (Hunter, 1949). Bearing in mind the possibilities of competition between coliform organisms and staphylococci (Parmala, 1956), I have listed the milk samples which showed low counts and failed the coliform test and which contained large numbers of Staphylococcus aureus (Table No. 7 page 55).

Table No. 7 Incidence of Low-Count, High-Coliform milks and the presence of Staphylococcus aureus in "Certified" Milk for 1956-57

	1956-57	
Season	Number with low count and high coliform content	Number of these with Staph. aureus
Winter	4	1
Spring	2	1
Summer	0	0

It /

It can be seen that only 1 of 4 and 1 of 2 of these samples had large numbers of Staphylococcus aureus present. I would conclude from these results that competition between coliforms and staphylococci is not responsible for low count, high coliform milks.

(b) Pasteurised Milk

The plate count is not one of the standard tests applied to pasteurised milk. Presumably most bacteria will have been destroyed by pasteurisation, and counts will be very low, or will tend to be composed of a selected population of heat resistant organisms e.g., spore formers or thermophilic species. Colony counts were carried out on pasteurised milk to compare it with "Certified" milk, and to compare the positive coliform tests with the plate counts. The same standard level of 30,000/ml. was chosen for comparison (Table No. 8, page 57).

From the table it can be seen that even in winter quite a large number (18.5%, 7.4%) of pasteurised milks have counts of over 30,000/ml. The proportion rose rapidly during summer and approximately half of the samples examined had high plate counts. The seasonal increase suggests insufficient cooling after the milk has been filled into improperly cleaned bottles. Subsequent bacterial multiplication has led to increased plate counts.

Table No. 9,(page 58) shows the number of samples failing the presumptive coliform test and with counts of over 30,000/ml.

It can be seen that most samples failing the coliform test have high plate counts. The incidence of low plate count with a positive coliform test is however slightly more common than with "Certified" milk. This is to be expected if most of the bacterial population has been killed by the temperatures of pasteurisation.

Part 3. /

Table 8 - Plate counts at 37°C of Pasteurised Milk for the years 1956-58

	1956-57			1957-58		
Season	Number of samples examined	Number over 30,000/ml.	% over 30,000/ml.	Number of samples examined	Number over 30,000/ml.	% over 30,000/ml.
Winter	27	5	18.5	27	2	7.4
Spring	19	8	42	23	2	8.6
Summer	22	8	36.6	19	9	47.3

Table 9 - Comparison of samples failing Presumptive Coliform Test and with counts of over 30,000/ml. for Pasteurised milk 1956-58

	1956-57		1957-58	
Season	Number failing coliform test	Number failing coliform test and count over 30,000/ml.	Number failing coliform test	Number failing coliform test and count over 30,000/ml.
Winter	1	1	2	1
Spring	4	3	0	0
Summer	6	5	8	5

Part 3

The Incidence of Clostridium welchii in Milk Supplies

Samples of consumer milk were examined for the presence of Clostridium welchii.

Procedure

This is described in the section on methods (page 32). Reactions were read as positive when typical stormy fermentation was obtained in litmus milk. In some cases it was doubtful if the "stormy clot" was typical although stormy fermentation was present. Positive tubes were re-inoculated into litmus milk to see if the stormy clot could be reproduced, and Gram-stained films were examined for Gram-positive rods. Robertson's meat medium was inoculated from the litmus milk and incubated anaerobically for 24 hr. at 37°C in a McIntosh and Fildes jar. The resulting growth was plated on to blood agar and incubated anaerobically for another 24 hr. Colonies from blood agar were examined and Cl. welchii identified by sugar fermentations and the Nagler reaction. Nagler plates were prepared by adding 3ml. of human serum to 10ml. of nutrient agar. Specific Cl. welchii type A antitoxin was spread over one half of the plate, and the surface dried in the incubator. The organisms under investigation were then streaked over the surface of both halves of the plate which was incubated anaerobically at 37°C. Cl. welchii produces the enzyme lecithinase which releases fat globules from lecithin in human serum. On a serum agar plate colonies of Cl. welchii are surrounded by a turbid halo (Plate 2B page 132). Presence of specific antitoxin neutralises lecithinase and no halo is seen. This was used as the final test of identification for Cl. welchii.

Results

In the years 1956-58, a total of 321 milk samples were examined /

examined for the presence of Cl. welchii. Of these, 13 samples were recorded as showing "stormy clots". (Table 10, page 61).

From the table it can be seen that Cl. welchii was present in both pasteurised and unpasteurised milk samples. Of the 13 "stormy clots" however, only 4 actually contained Cl. welchii. In another case (sample 37) another sporing anaerobe was isolated but repeated anaerobic culture failed to show clostridia in any of the other samples.

Organisms responsible for false "stormy clots" included lactobacilli, a combination of E.coli and streptococci, and B.subtilis (in the absence of coliform organisms). The fact that one of the pasteurised samples (21) had a positive coliform test may mean that pasteurisation was not efficient or that contamination had followed heat treatment. It would appear that several types of sporing organisms can survive pasteurisation eg. samples 21, 24, 2M, 2Q. These results also show that the standard quick test for recognising Cl. welchii by a "stormy clot" is not valid. Convincing stormy clots can be produced by entirely different organisms alone, or synergistically with others (eg. sample 1M plate 3, page 133, sample 2M plate 4, page 134, samples h and r, plate 5, page 135).

The presence of Cl. welchii in milk is not valid as a test for faecal contamination, because there are many sources of this organism on the farm and being a sporing organism, it can survive temperatures of pasteurisation.

The only significance that it might have would be if the types known to be responsible for food poisoning were present in large numbers in milk and could grow freely before the milk was consumed. Hobbs et al. (1953) have made extensive investigations into the types of Cl. welchii isolated from outbreaks of food poisoning and have described their physiological and biochemical properties. /

Table 10 - Organisms Responsible for "Stormy Clots" in Litmus Milk

1956-57	Sample labelled	Type of milk	Organisms isolated
	21	Pasteurised	Cl. welchii
	23	"Certified"	Cl. welchii
	37	T.T. Unpasteurised	(Sporing anaerobe and (Lactobacillus
	38	T.T. Unpasteurised	Lactobacillus
	1M	T.T. Unpasteurised	(Lactobacillus and (Micrococci
1957-58	24	Pasteurised	Cl. welchii
	A	"Certified"	Cl. welchii
	C	"Certified"	Streptococci (stormy clot not reproducible)
	2M	Pasteurised	B.subtilis
	2Q	Pasteurised	B.subtilis
	h	"Certified"	E.coli and Streptococci
	r	"Certified"	Streptococci and E.coli
	t	"Certified"	Streptococci

properties. Morphologically they are typical Gram-positive rods, with spores rarely present. They are however non-haemolytic on blood agar on primary isolation. Biochemically they resemble other strains of Cl. welchii - i.e. they ferment glucose, lactose, maltose, and sucrose to give acid and gas and produce a "stormy clot" in litmus milk. They will give a positive Nagler reaction, inhibited by type-A antitoxin because both these organisms produce α toxin. The food-poisoning variants are in fact very similar to typical type-A strains except for their greater heat resistance and their non-haemolytic appearance on blood agar, which is due to their inability to produce θ toxin.

Of the cultures isolated, only one (from sample 24) gave possibly non-haemolytic colonies. As this was a pasteurised milk, and food poisoning strains are heat resistant, it is possible that this was one. No attempts were made to determine the actual toxins possessed by the culture apart from inhibition of the Nagler reaction by type-A antitoxin.

On the whole it would seem unlikely that the presence of Cl. welchii in milk is a source of food poisoning.

Part 4

Production of False Positive Coliform Reactions

A few experiments were carried out with combinations of two organisms to see whether by synergistic reactions they could produce acid and gas in MacConkey broth. The principle behind the choice of organisms used was the ability of one to ferment sugars - especially lactose - to give acid, probably through glucose and galactose as intermediates. This would be associated with the ability of the other organism to ferment either glucose or galactose to give acid and gas. Organisms were chosen which were likely to be found in milk, viz:-

Streptococcus /

Streptococcus lactis
" cremoris
" faecalis
" bovis
" agalactiae
" thermophilus

Staphylococcus aureus
Bacillus subtilis
" cereus
" polymyxa (sometimes responsible for false positives in water analysis.)

These organisms all ferment lactose to give acid only. Those splitting glucose to give acid and gas were:-

Proteus vulgaris
Clostridium welchii
" sporogenes

A few drops of an overnight broth culture of the organisms were added in pairs to tubes of MacConkey broth containing Durham tubes, and the tubes were incubated at 37°C for 48 hr. A control experiment was set up consisting of MacConkey broth containing only one organism. Results are shown in Tables 11,(page 64) and 12, (page 65).

From Table 12 it can be seen that only the lactic and faecal streptococci, Staph. aureus, and B. polymyxa grew in MacConkey broth alone.

Synergistic reactions in MacConkey broth giving rise to acid and gas were obtained only with P. vulgaris and Strep. faecalis, Strep. cremoris, or Staph. aureus. A synergistic reaction was observed between Cl. sporogenes and Strep. agalactiae although neither grew alone in MacConkey broth.

Because many of the false positives were obtained in the tubes which received 1ml. of undiluted milk in the standard test, it was decided to repeat the above experiments with the same organisms, but to add 1 ml. of undiluted milk (autoclaved at 10 lb./sq. inch) to /

Table 11 Growth of Pure Cultures of One Organism in MacConkey Broth

(+ = growth A = acid)

Organism	Growth	Organism	Growth
Strep. lactis	+A	Staph. aureus	+A
Strep. cremoris	+A	B. subtilis	-
Strep. faecalis	+A	B. cereus	-
Strep. agalactiae	-	B. polymyxa	+A
Strep. bovis	+A	Cl. welchii	-
Strep. thermophilus		Cl. sporogenes	-
Proteus vulgaris			

Table 12 Results of Adding Pairs of Organisms to MacConkey Broth

Producing acid and gas from glucose	Fermenting lactose to acid	Reaction in MacConkey Broth
Cl. welchii	Strep. lactis	Acid
	Strep. cremoris	Acid
	Strep. faecalis	Acid
	Strep. bovis	Acid
	Strep. agalactiae	-
	Staph. aureus	Acid
Cl. sporogenes	Strep. lactis	Acid
	Strep. cremoris	Acid
	Strep. faecalis	Acid
	Strep. bovis	Acid
	Strep. agalactiae	Acid and gas (small vol.)
	Staph. aureus	Acid
Proteus vulgaris	Strep. lactis	Acid and gas (small vol.)
	Strep. cremoris	Acid
	Strep. faecalis	Acid and gas (small vol.)
	Strep. bovis	Acid
	Staph. aureus	Acid and gas (small vol.)
Bacillus polymyxa	Strep. lactis	-
	Strep. cremoris	Acid
	Strep. faecalis	Acid
	Strep. bovis	Acid
	Strep. agalactiae	Acid
	Staph. aureus	Acid

to the tubes. A control was set up containing only one ml. of milk. Table 13 (page 67) shows the growth of organisms in MacConkey broth with 1 ml. of sterile milk.

Control tubes containing only 1 ml. of milk showed no acid or gas production. Table 14 (page 68) shows the effect of adding 1 ml. of sterile milk to pairs of organisms in MacConkey broth.

It can be seen from these results that addition of 1ml. of milk affects the selectiveness of the MacConkey broth. All organisms can grow when 1ml. of milk is present, and in addition, Cl. welchii, Cl. sporogenes, P. vulgaris and B. subtilis will produce gas in the Durham tube. Similarly, addition of milk increases the false positives obtained by adding two organisms to MacConkey broth (Table 14). The organism which produced gas in the Durham tubes most often, either alone, or in combination with another organism, was B. subtilis. Now this organism does not ferment lactose, but ferments glucose to give acid only. Therefore gas production is not due to sugar fermentation. B. subtilis liquefies gelatin, peptonises milk, and hydrolyses casein. This would account for the alkaline reaction in the tube, the casein of the milk being split to amino acids. Possible decarboxylation of the amino acids might give rise to the gas volume in the Durham tube. Indeed it is known (Waksman, 1952) that B. subtilis can break down casein to give about 46 mg. of amino-nitrogen and the process of ammonia formation is completed in a few days.

The other most prominent organisms in false-positive tests appear to be Cl. welchii and Cl. sporogenes. These organisms will split lactose to give acid and gas, but do not grow in MacConkey broth alone in aerobic conditions. Addition of 1ml. of milk enriches the medium and possibly creates anaerobic conditions when the cream settles as a layer on the surface of the medium. Synergistic reactions are /

Table 13 Growth of Pure Cultures in MacConkey Broth with 1 ml. of Sterile Milk

(+ = growth A = acid G = gas)

Organism	Growth	Organism	Growth
Strep. lactis	+ A	Staph. aureus	+A
Strep. cremoris	+ A	B. subtilis	+G (alkaline)
Strep. faecalis	+ A	B. cereus	+A
Strep. bovis	+ A	B. polymyxa	+A
Strep. agalactiae	+ A	Cl. welchii	+AG
Strep. thermophilus	+ A	Cl. sporogenes	+AG
		P. vulgaris	+AG

Table 14 Acid and Gas Production in MacConkey Broth with 1ml. of sterile milk due to Synergistic Actions

Producing acid and gas in glucose	Fermenting lactose to acid	Reaction in MacConkey
Cl. welchii	Strep. faecalis	Acid
	Strep. lactis	Acid
	Strep. cremoris	Acid
	Strep. agalactiae	Acid
	Staph. aureus	Acid
	Bacillus subtilis	Acid and Gas
Cl. sporogenes	Strep. faecalis	Acid
	Strep. lactis	Acid
	Strep. cremoris	Acid and Gas (small vol.)
	Strep. agalactiae	Acid and Gas
	Staph. aureus	Acid and Gas
	Bacillus subtilis	Gas, Alkaline
Proteus Vulgaris	Strep. faecalis	Acid
	Strep. lactis	Acid
	Strep. cremoris	Acid
	Strep. agalactiae	Acid
	Staph. aureus	Acid and Gas (small vol.)
	Bacillus subtilis	Acid and Gas (small vol.)
Bacillus Polymyxa	Strep. faecalis	Acid
	Strep. lactis	Acid
	Strep. cremoris	Acid
	Strep. agalactiae	Acid
	Staph. aureus	Acid

are not very common with the lactic acid bacteria, e.g. Strep. lactis and Strep. cremoris, probably because the extremely high concentration of acid produced will inhibit other gas-forming bacteria.

On the whole it would seem that false positive presumptive coliform tests are unlikely to affect the value of the statutory test. They occur most often in the tubes receiving 1ml. of milk, and these are not the critical ones which fail milk samples. In higher dilutions, organisms causing false positives are not enriched to the same extent, and in the case of B. subtilis, the tube becomes alkaline instead of acid.

Part 5

The Use of Tetrazolium Salts in Media for Quicker and More selective Presumptive Coliform Tests

It is well known that tetrazolium salts have a wide application in biological laboratories (B.D.H. Ltd. Manufacturer's Review, 1958). In bacteriology they are well known for their vital staining properties. Living cells will reduce the salts to coloured formazans, which can be seen in the cells. As already described, attempts have been made to use one of these salts as a selective inhibitor in a solid medium for quick detection of E.coli (Chapman, 1951). Other workers have described the use of triphenyltetrazolium chloride broth for the quick detection of coliform organisms in milk and water (Schönberg, 1954; Kraus, 1959). It is claimed that a concentration of 0.22% triphenyltetrazolium chloride (T T C) in nutrient broth inhibits most organisms but will allow coliforms to grow, giving reduction of the tetrazolium salt to a red formazan within 6 hr. Chapman, however, states that although most coliforms reduce T T C, E.coli does not although it is not inhibited by T T C.

I decided, therefore, to try to verify one or other of these conflicting /

conflicting statements, and to investigate the possibility of employing a T T C broth for the quick detection of coliforms in milk, to replace the 48 hr. incubation period required with MacConkey broth.

Materials and Methods

The medium employed was the acid nutrient broth suggested by Schönberg (1954) and Kraus (1959), and had the following composition:-

Minced ox heart muscle	500 gm.
Peptone ("Oxoid")	10 "
Sodium Chloride	5 "
Water	1,000 ml.

pH 6.4

Sterilised by autoclaving at 10lb./sq.in. for 20min.

Solutions of triphenyltetrazolium chloride were prepared in glass-distilled water and sterilised by Seitz filtration. The tetrazolium salt was obtained from B.D.H. Ltd.

Later, a few experiments were carried out with the acid broth supplemented with 0.5% lactose. Tubes of broth containing 0.22% T T C were inoculated with a drop of an overnight broth culture of the organisms under study. These were organisms which might possibly be present in milk, in addition to a selection of coliforms. Results are shown in Table 15 (page 71).

A control tube of uninoculated T T C broth was incubated in the same way to confirm that the T T C salt was not reduced by incubation alone.

From these results it can be seen that almost all the organisms tested will grow in 0.22% T T C broth, and will reduce the salt to a red formazan. E.coli grew but in this case did not reduce the T T C.

It would seem that a concentration of 0.22% T T C is not selective for coliforms.

A /

Table 15 Growth and Reduction in 0.22% T T C Broth Inoculated with Pure Cultures of Bacteria

\+ = growth R = reduction of T T C

Organism	Reaction	Organism	Reaction
E.coli N.C.T.C. 1093		Staph. aureus	+R
K.aerogenes	+R	Strep. pyogenes	+R
Intermediate coliform	+R	Strep. bovis	+R
P. vulgaris	+R	Strep. faecalis	+R
Serratia sp.	+R	Cl. welchii	-
B.subtilis	-	Cl. sporogenes	-

A further series of experiments was conducted in which a solution containing 20mg./ml. of T T C was added to broth in appropriate amounts to give different concentrations of T T C, and the growth of the same organisms was compared at these different concentrations after 24 hr. at 37°C (Table 16, page 73).

The results of this experiment show that most of the organisms will grow in much higher concentrations of T T C than 0.22%. Organisms of the coliform group and related organisms, K. aerogenes, Intermediate coliforms, P. vulgaris, Serratia sp.) will grow in high concentrations (2.5, 5mg./ml.) of T T C. Conversely, E.coli is inhibited by a concentration of over 2 mg./ml. This is true for two strains of the organism tested. In this experiment, in contrast to the previous, both strains of E.coli reduced the salt to a red formazan. It can be seen that organisms other than coliforms can grow at a concentration of 2mg./ml. (Approx. 0.22%) of T T C, e.g. Strep. faecalis, Staph. aureus, P. vulgaris and Serratia sp. If milk (or water) were to be inoculated into such a medium, reduction of the T T C could not be taken as an indication that coliforms organisms were present as the reaction could be given by any of the above-mentioned organisms.

Of the organisms growing at this concentration of T T C, however, only the coliforms will ferment the sugar lactose to give acid and gas. The others are either non-lactose fermenters or will give an acid only fermentation of the sugar. I thought it might be of interest to see what type of reactions were obtained in a lactose broth containing these concentrations of T T C. Included in the tubes of broth were Durham tubes to record gas fermentation of the sugar. As before, suitable amounts of a stock solution (20mg./ml.) of T T C were added to 6ml. amounts of broth and the tubes were inoculated with different organisms and incubated at 37°C for 18-24 hours. /

Table 16 Growth of Different Organisms in Various Concentrations of T T C

\+ = growth R = Reduction

Organism	Concentration of T T C (mg./ml.)				
	1.0	1.25	2.0	2.5	5.0
E.coli 1093	+R	+R	+R	-	-
E.coli (milk isolate)	+R	+R	+R	-	-
K.aerogenes	+R	+R	+R	+R	+R
Intermediate coliform	+R	+R	+R	+R	+R
P.vulgaris	+R	+R	+R	+R	-
Serratia sp.	+R	+R	+R	+R	+R
Staph. aureus	+R	+R	+R	-	-
Strep. pyogenes	-	-	-	-	-
Strep. bovis	+R	+R	-	-	-
Strep. faecalis	+R	+R	+R	-	-
Cl. welchii	-	-	-	-	-

hours. Results were very inconsistent and the figures for four experiments are shown in Tables 17 a and b, (page 75) and c and d, (page 76).

From these results it can be seen that K.aerogenes and the intermediate coliform will produce gas in lactose broth containing up to 5mg./ml. of T T C P.vulgaris will grow up to this concentration and will reduce T T C but will not produce gas. Results with the two strains of E.coli are inconsistent. In three of the four experiments E.coli 1093 gave gas up to 4mg./ml. and reduction of T T C but no gas at 5mg./ml. In the other experiment it grew and gave gas only up to 3.3mg./ml. The other strain, tested only three times, gave gas up to 4mg./ml. and reduction only at 5mg./ml.

Results with Staph. aureus and Strep. faecalis were extremely irregular.

From these results it would seem that a concentration of 4mg./ml. of T T C would be more selective for coliforms. Because of the ability of other organisms, e.g. P.vulgaris and Staph.aureus to grow at this concentration, and to give reduction of T T C, it is advisable to carry out the tests in a lactose broth and to measure gas formation in a Durham tube. Altogether this method of testing for coliforms in water or milk seems very inferior to the standard MacConkey broth method. The results are too variable, and may be very misleading.

SECTION /

Table 17a Production of acid and gas in lactose broth containing different concentrations of T T C

+ = growth R = reduction to formazan G = gas

Organism	Concentration of T T C (mg./ml.)					
	1.0	2.0	2.5	3.3	4.0	5.0
E.coli 1093	+ R	+R	+RG	+RG	+RG	
K.aerogenes	+ RG	+RG	+RG	+RG	+RG	
Intermediate coliform	+ RG	+RG	+RG	+RG	+RG	
P.vulgaris	+ R	+R	+R	+R	+R	
Staph. aureus	+ R		+R	+R		
Cl.welchii	-	-	-	-	-	

Table 17b

E.coli 1093	+RG	+ R
E.coli (Milk isolate)	+RG	+ RG
K.aerogenes	+RG	+ RG
Intermediate coliform	+RG	+ RG
P.vulgaris	+R	+ R
Staph. aureus	+R	+ R
Strep. faecalis	+R	+ R

Table 17 c Production of acid and gas in lactose broth containing different concentrations of T T C

+ = growth R = reduction to formazan G = gas

Organism	Concentration of T T C (mg./ml.)					
	1.0	2.0	2.5	3.0	4.0	5.0
E.coli 1093	+RG	-	+ R	+RG	-	-
E.coli (Milk isolate)	+RG	+ RG	+ RG	+RG	+ RG	+ R
K.aerogenes	+RG	+ RG	+ RG	+RG	+ RG	-
Intermediate coliform	+RG	+RG	+RG	+RG	+ RG	+RG
P.vulgaris	+R	+ R	+R	+R	+ R	+R
Staph. aureus	+ R	+ R	-	-	-	-
Strep. faecalis	+R	+ R	+R	+R	+R	-

Table 17d

E.coli 1093			+RG	+RG	+RG	+R
E.coli (Milk isolate)			+RG	+RG	+RG	+RG
K.aerogenes			+RG	+RG	+RG	+ RG
Intermediate coliform			+RG	+RG	+RG	+RG
P.vulgaris			+R	+ R	+R	+ R
Staph. aureus			+ R	+R	+R	+R
Strep. faecalis			+ R	+ R	-	+ R
Cl. welchii	-	-	-	-	-	-

SECTION II

Incidence of Staphylococcus aureus in Consumer Milk

Introduction

During the examination of milks of different grades, I noticed that many of the milk agar plates obtained in the plate counts showed typical colonies of Staphylococcus aureus. On further examination these were found to be coagulase-positive; and some were resistant to penicillin. I decided therefore to examine all milk agar plates for Staph. aureus and to determine the penicillin resistance of the cultures isolated.

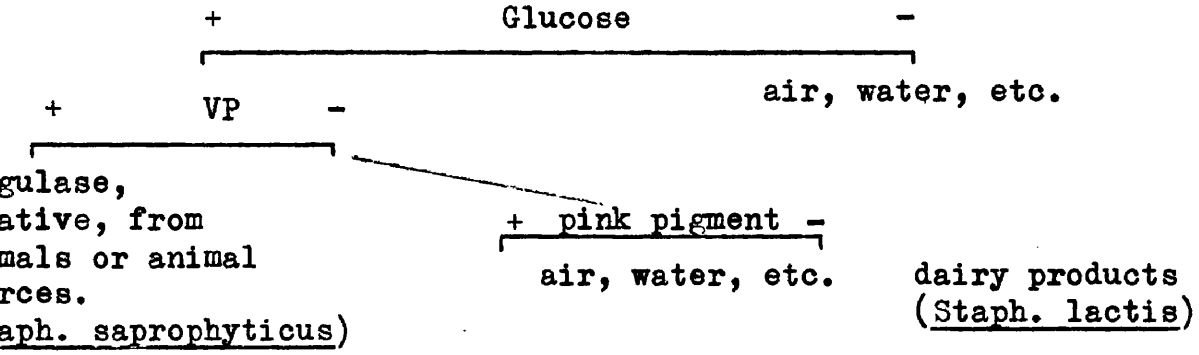
Staph. aureus is not an uncommon organism in milk. The organism may enter the milk from the hands or nasopharynx of dairy workers who carry it, from dirty bottles, and from cows suffering from mastitis. Before 1941, most cases of mastitis were due to streptococcal infections but these are now outnumbered by staphylococcal ones (Laing and Malcolm, 1956). It has become the practice of farmers to treat cows suffering from mastitis with penicillin; this, as in human medical practice, especially in hospitals, may give rise to penicillin-resistant staphylococci. Some farmers treat their own cows without seeking advice on dosage, and an insufficient course of antibiotic therapy is employed. This is especially likely to give rise to resistance.

In a study of coagulase-positive staphylococci isolated from bovine mastitis, Prïce et al., (1954) found infections caused by penicillin-resistant staphylococci in quarters of udders which had not been treated with penicillin. Indeed, the appearance of penicillin-resistant Staph. aureus in milk is not rare even without antibiotic treatment, although this certainly helps to select resistant strains in milk. There is a large number of cocci in milk. Because of some confusion in nomenclature of the Gram-positive cocci, /

cocci, we have names such as Staphylococcus and Micrococcus for the genus; there is no really accepted means of separating the different species of staphylococci or micrococci.

But one species seems fairly well defined, and stands apart from the others i.e. Staphylococcus aureus Rosenbach. The possession by this organism of the enzyme coagulase, which clots human plasma, distinguishes it from other staphylococci.

Attempts have been made to subdivide further the remaining staphylococci, but for most people the primary division is into coagulase-positive and coagulase-negative types. Shaw, Stitt, and Cowan (1951) evolved a scheme of classification as follows:- The first clear division is into coagulase-positive and coagulase-negative types. The coagulase-positive group is homogenous, containing toxigenic and pathogenic strains, sharing a series of common antigens and sensitive to a series of specific bacteriophages. The coagulase-negative group was further subdivided by criteria which seldom showed variation as follows:-



Bergy's Manual (6th edition) (1948) on the other hand, uses pigment production as primary significance, but this is known to be variable with most organisms. The seventh edition (1957), divided the original genus into two genera:-

1) Micrococcus, containing free-living organisms, able to utilise simple inorganic carbon and nitrogen compounds.

2)

2) Staphylococcus, containing only two species, one being the coagulase-positive, mannitol-fermenting, enterotoxin-producing, Staph. aureus.

The generic name Staphylococcus and the nomenclatural type species Staphylococcus aureus Rosenbach 1884, have now been officially conserved. (Int. Comm. on Bact. Nom., 1958.)

Thatcher and Simon (1957) concluded that neither the Voges Proskauer test, the anaerobic production of acid from glucose, nor the production of the enzyme phosphatase, are related to homogeneous groups of staphylococcus. For the present purpose, it was required to be able to isolate and identify the organism known as Staph. aureus, which is regarded as a potential or immediate pathogen. Various media have been evolved for the rapid isolation of pathogenic Staph. aureus. Among these, is the mannitol, salt agar suggested by Chapman (1945). The medium contains 7.5% sodium chloride, and coagulase-positive staphylococci grow well, the colonies being surrounded by a yellow zone. Other coagulase-negative staphylococci give small colonies surrounded by red or purple zones.

Milk agar is known to favour the golden-yellow pigmentation of Staph. aureus. A test has been developed for the rapid detection of potentially pathogenic strains of Staph. aureus (Barber and Kuper, 1951, White and Picket, 1953). This depends on the possession by the organisms of the enzyme phosphatase. Of 20 coagulase-positive strains studied by White and Picket, all were phosphatase-positive, and all of 13 coagulase-negative strains were phosphatase-negative.

A medium was described by Taylor and McDiarmid (1948) for the detection of coagulase-positive staphylococci of bovine origin. A similar medium was evolved for the rapid recognition of coagulase-positive, penicillin-resistant staphylococci by Klemperer and Haughton (1957). Human plasma, bovine fibrinogen, and penicillin are incorporated into agar. Opacity develops round coagulase-positive colonies, /

colonies, and only penicillin-resistant organisms will grow. There was complete correlation between opacity surrounding colonies and a positive tube-coagulase test. The standard test for pathogenic Staph. aureus is the coagulase test. Most authors of bacteriological textbooks (Topley and Wilson, Mackie, Burrows) and other bacteriologists regard a positive coagulase test as the best single criterion of pathogenicity. Coagulase activity is demonstrated by the clotting of oxalated or citrated plasma. It is thought that coagulase-positive staphylococci are less susceptible to phagocytosis, in presence of plasma, than are coagulase-negative strains because the cells are protected by the clot. It aids initial development and formation of lesions, then α-toxin is produced (Hale and Smith, 1945). Chapman et al. (1934) confirmed the importance of the coagulase test. They also emphasized that although a golden-yellow pigment was often characteristic of Staph. aureus and aureus strains are usually more pathogenic than albus or white strains, pigment is not a reliable guide to pathogenicity as pigment production may vary immensely.

Haemolysis on rabbit blood agar is often used as a criterion of pathogenicity but Chapman et al. pointed out that some pathogenic strains are non-hameolytic; in a series of 690 strains of Staph. aureus examined by them, only 51.7% were haemolytic. It is important that haemolysis should be studied on rabbit blood agar, and the confusing accounts of haemolysis of staphylococci may be due to use of other types of blood agar. The coagulase test, if carried out carefully in standard conditions is the best indication of pathogenic staphylococci. Coagulase may be detected in two ways viz., (1) slide test; (2) tube test. In the former, a loopful of culture from a solid medium is emulsified in plasma on a slide and if cells are coagulase-positive, clumping occurs. The tube test consists of incubating standard amounts of broth culture of the organism and suitably /

suitably diluted plasma at 37°C and examining at intervals for clotting.

Duthie (1954) pointed out that two forms of coagulase are produced by staphylococci. One is bound to the cell wall, and is responsible for the slide test, the other is liberated as free coagulase into the medium and is responsible for the tube test. Both types are antigenically distinct. The coagulase test must be carried out in controlled conditions, since plasma from different animals may show different results with the same organism. It was also shown by Lominski et al., (1953) that some weakly coagulase-positive, and apparently coagulase-negative, strains of Staph. aureus produce not only coagulase but also a factor capable of destroying coagulase.

Biochemical tests have no great significance in the differentiation of staphylococci. However the fermentation of mannitol, with the production of acid without gas, and the liquefaction of gelatin are usually regarded as indications of pathogenic strains of Staph.aureus (Zinsser, 1948, Mackie, 1953).

A more reliable criterion of pathogenic Staph. aureus may be the production of toxins i.e. α and β toxins and enterotoxin. Indeed Thatcher and Simon (1957) described strains of Staph. aureus which produced toxin but were coagulase-negative. This may be due to destruction of free coagulase, and thus a negative tube test, whereas the organism may still give a positive slide-coagulase test.

The differentiation of the species Staph. aureus into different types has now been made possible by bacteriophage typing (Fisk, 1942, Wilson and Aitkinson, 1945, Williams and Rippon, 1952.) Only coagulase-positive staphylococci may be typed by this method, each type of phage being specific for a certain antigenic type of cell. Indeed phage typing has shown that pigment production is not necessarily /

necessarily an indication of similarity (Fisk, 1942). Identical phage types have been isolated which differed in pigment production.

In this thesis, all future references to Staph. aureus will mean coagulase-positive staphylococci, regardless of lack of haemolysis on blood agar. Coagulase-positive organisms failing to produce the golden-yellow pigment will be called Staph. aureus var. albus.

STAPHYLOCOCCI IN MILK

Staphylococci are commonly found in milk. In cases of staphylococcal mastitis, the cow may excrete large numbers of organisms into the milk. When large numbers of such organisms are isolated, the question arises are these pathogenic Staph. aureus likely to be harmful to anyone consuming the milk, especially young children? If this is found to be so, and the organisms are penicillin-resistant, the significance is clearly serious. Staphylococcal mastitis is much more common to-day than previously. There is also a condition known as "non-specific" mastitis, where the milk shows high leucocyte counts comparable to quarters infected with mastitis, but no organisms are isolated from the condition. From some such milks, staphylococci have been isolated but have been found to be coagulase-negative (Miss C.M. Laing, personal communication). Attempts have been made to type the coagulase-positive staphylococci from cases of bovine mastitis, to determine whether they are types which are normally associated with pathogenic conditions in man. MacDonald (1946) typed 150 strains of Staph. aureus obtained from accredited milk and 34 strains from cases of bovine mastitis. Of the milk strains 123 of the 150 were susceptible to phage 42D and all the mastitis strains were of phage-type 42D.

Williams Smith (1948) carried out a similar survey and also came to the conclusion that phage typing did not distinguish strains of Staph. aureus isolated from cases of mastitis from strains isolated from normal udders. Nevertheless, he isolated 3 strains of Staph. aureus, all type 42D, which were antigenically identical and which came from 3 significant sources viz. a case of staphylococcal food poisoning, a case of human mastitis, and a case of bovine mastitis. All strains were able to maintain themselves for 6 weeks in the bovine udder and this suggested that some strains of type 42D may be capable of infecting man as well as cows.

Price et al. (1954) studied staphylococci isolated from cases of bovine mastitis before and after treatment with antibiotics. They again found the commonest phage type to be 42D, but 42B was also common. Many of the penicillin-resistant organisms isolated came from quarters of udders not treated with antibiotics, indicating that antibiotic treatment alone is not responsible for the appearance of resistant organisms. These workers also suggested that as the phages used for identifying the staphylococci were from strains of human origin, it was probable that a more satisfactory result would be obtained if phages were from staphylococci of bovine origin.

Jones et al., (1957 dealt with the growth of Staph.aureus in milk and its possible connection with food poisoning. They concluded that food poisoning from fresh raw milk was unlikely, unless 4 conditions were fulfilled - namely:-

(1) almost complete absence of organisms which might overgrow Staph. aureus;
(2) storage at a fairly high temperature e.g. 37°C for some time;
(3) the milk must not be mixed with other milk because this would dilute the toxin;
(4) the milk must not be submitted to temperatures which would destroy /

destroy the toxin.

Thus it is unlikely in practice that milk even from cows suffering from Staph. aureus mastitis will cause food poisoning, even if the staphylococci are of types pathogenic to man.

Staph. aureus is a potential human pathogen. Some strains produce enterotoxin - a toxin causing food poisoning. If a milk contains large numbers of such organisms, there may also be enterotoxin present if the milk has been produced and stored in such a way as to allow the staphylococci to grow. Milk being a favourable growth medium for staphylococci, they can multiply and produce toxin, although Jones et al. (1957) state that even in favourable conditions there is little produced. Staphylococcal food poisoning from dried milk is not uncommon (Anderson and Stone, 1955; Hobbs, 1955), although this may be due to contamination after dehydration. In one case phage type 42E/53+ was isolated. Clean milk, as purchased by the consumer should not contain large numbers of Staph. aureus. This organism may gain entrance to milk from:-

(1) milk cows suffering from mastitis due to Staph. aureus;
(2) from hands or nasopharynx of milkers or dairy workers; and
(3) from unclean utensils, such as dirty bottles and milking machines.

Staphylococcal infections are still usually treated, especially outside hospitals, with the antibiotic penicillin. In recent years, however, increasing numbers of penicillin-resistant staphylococcal infections are appearing (see, for example, McDermott, 1956). Bovine mastitis treated with penicillin may select penicillin-resistant staphylococci, and these are in general, undesirable additions to milk. It seemed worth while finding out how often and in what order of numbers they were present.

During my examination of different grades of milk as bought by the consumers the routine plate count was carried out. When first examining /

examining milk of "Certified" grade, I noticed that there were large numbers of golden-yellow colonies (very similar to colonies of Staph.aureus) on the plates. On further examination they indeed proved to be Staph. aureus, and were tested for their sensitivity to penicillin. It was found that some of the organisms were penicillin-resistant and so, in addition to the other investigations of milk, it was decided to examine all milk agar plates for colonies of Staph. aureus and to determine the penicillin-sensitivity of such organisms.

Sources of milks

Milks were of different grades, bottled as bought by consumer. Three types were examined, (1) Pasteurised; (2) "Certified"; and (3) Tuberculin tested (T.T.), either pasteurised or unpasteurised.

At the same time, Miss C.M. Laing of the West of Scotland College of Agriculture was examining milk samples from cases of suspected mastitis, and had isolated many cultures of staphylococci, some of them penicillin-resistant. It was decided, therefore to test these cultures for their degree of resistance to penicillin, and to have them typed by bacteriophage, to see whether they were of phage types normally found in human infections.

Material and Methods

Isolation

Golden-yellow, typical staphylococcal colonies were picked up from milk agar plates and inoculated into meat extract broth incubated for 4-5 hours at 37°C and used for coagulase test and penicillin-sensitivity tests. Smears were made from broths and stained by Gram's method to check the presence of Gram-positive cocci. Selective media, such as mannitol salt agar and plasma agar, were not used /

used as there was no difficulty in recognising typical colonies on milk agar, which is known to enhance pigment production by Staph. aureus.

Methods

1. Coagulase test: The tube test was employed. A 1 in 10 dilution of human plasma in sterile saline was used (0.1ml. of plasma plus 0.9ml. saline) and to this a few drops of a 4-5 hour broth culture of staphylococci were added and the tubes incubated for 3 hours at 37°C. Tubes were then examined half-hourly for coagulation. If coagulation was not apparent after 4 hours the strain was regarded as being coagulase-negative. The plasma used had been stored in a deep-freeze cabinet and was thereafter kept in a refrigerator before use in the test. A strongly positive reaction was indicated by a solid clot and weaker reactions included "balloons" and soft filmy clots. Negative tubes were not left overnight on the bench and thereafter read, because this type of coagulation may not be due to coagulase production. It has been shown that utilisation of citrate in citrated plasma by various organisms, e.g. E.coli, leads to a positive reaction in 8-10 hours (Mushin and Kerr, 1954.).

2. Biochemical tests: In addition to coagulase tests, the cultures isolated were also tested for the ability to ferment mannitol and to liquefy gelatin. Tubes of 1% solution in peptone water were incubated for 24 hours at 37°C. Similarly, stab inoculations of gelatin deeps were incubated in the same way, and if they had not liquefied in 24 hours were kept at room temperature for 2-3 weeks before they were classed as negative.

3. Penicillin sensitivity: A few drops of a 4-5 hour broth culture were spread over the surface of a nutrient agar plate and tablets of antibiotics dropped on the surface. Sensitivity to a given antibiotic /

antibiotic was indicated by a zone of no growth round the tablet (Plate 7),(page 138). Organisms resistant to a particular antibiotic grew right up to the tablet (Plate 8),(page 139). All strains were tested for resistance to penicillin, and some were also tested for resistance to streptomycin, chlortetracycline and erythromycin. There were no strains isolated which were resistant to these two other antibiotics.

4. Degree of Penicillin sensitivity: The standard method of tube dilution was used in determining penicillin sensitivity. A series of doubling dilutions of a solution of penicillin containing 20.6 I.U. penicillin/ml. was made in 1 ml. amounts of meat extract broth. Penicillin solution was sterilized by Seitz filtration. To each tube was added one drop of a 1 in 300 dilution of an overnight broth culture of Staph. aureus and tubes were incubated overnight at 37°C. A control was also observed with the Oxford strain of Staph. aureus. Sensitivity to a given concentration of penicillin was shown by absence of growth in tubes. Growth and turbidity was observed in tubes where the organism could resist the concentration of penicillin present.

5. Phage typing: Phage typing was very kindly carried out by Dr. Morag Timbury of the Bacteriology Department of the Western Infirmary of Glasgow.

Milks were examined during a "winter" and "spring" period 1956-57. Types examined were "Certified", T.T., and Pasteurised, but staphylococci were never isolated from pasteurised milk during the experiment.

Results

1. "Certified" Milk: Table 18 (page 88) shows the number of milks examined and of coagulase-positive Staph. aureus isolated from "Certified" /

TABLE 18

Coagulase-positive Staph.aureus isolated from "Certified" Milk

WINTER			SPRING		
Sample No.	Number of colonies of coagulase + S.aureus isolated	No. of these penicillin resistant	Sample No.	Number of colonies of coagulase + S.aureus isolated	No. of these penicillin resistant
1	0	...	A	1	0
2	0	...	B	3	1
3	not examined		C	6	5
4	1	0	D	3	3
5	1	1	E	1	0
6	3	0	F	1	1
7	1	1	G	1	0
8	0	...	H	2	0
9	0	...	I	0	...
10	0	...	J	4	2
11	3	0	K	0	...
12	4	0	L	0	...
13	1	0	M	0	...
14	6	3	N	0	...
15	6	6	O	0	...
16	0	...	P	0	...
17	6	6	Q	2	no more resistance tests carried out
18	0	...	R	2	
19	0	...	S	0	
20	5	1	T	2	
21	0	...	U	0	
22	2	2	V	1	
23	3	3			
24	0	...			
24	13 (samples)	8 of 13+ samples	22	13 (samples)	5 of 9+ samples
%	54.1% (of samples)	61.5% (of + samples)	%	59% (of samples)	55.5% (of + samples tested)

... No relevant observation.

"Certified" milk during a winter and spring period. Not all colonies were picked from plates but a fair representative selection. Table 18 also shows how many of Staph. aureus were penicillin-resistant as tested by plate and tablet method.

During the winter period 13 of the 24 samples examined contained Staph. aureus (i.e. 54.1%). Of those containing Staph. aureus, 8 contained penicillin-resistant organisms (i.e. 61.5%). In two of the samples (Nos. 14 and 20) both penicillin-sensitive, and penicillin-resistant Staph. aureus were isolated. In sample 20, the resistant organism was of a different phage type from the others, whereas in sample 14, the resistant and sensitive types were of the same phage type. (Table 19, page 90). In the spring period, for samples A - P, 13 of the 22 samples had Staph. aureus (i.e. 59%). Of the 9 examined, 5 had penicillin-resistant Staph. aureus (i.e. 55.5%). For reasons explained later, it was decided at that point to abandon phage typing and penicillin-resistance determinations for the Staph. aureus strains isolated from milk. The phage types of the cultures isolated during the winter period are listed in table 19. The information is not complete, because phage typing was abandoned when it was found that the cultures were mainly 42D and had many cross-reactions.

As there has been much controversy about the biochemical reactions of potentially pathogenic Staph. aureus, the fermentation of mannitol and liquefaction of gelatin were noted when identifying the organisms. Table 20, (page 91) shows the results with coagulase-positive penicillin-resistant organisms and also their degrees of penicillin resistance.

From the results in table 20 it can be seen that all the coagulase-positive Staph. aureus isolated, fermented mannitol. Not all, however, liquefied gelatin (even after 3 weeks), 1 of 31 cultures /

Table 19 Phage Types of Staph. aureus isolated from "Certified" Milk

Milk Sample	Colonies No.	Penicillin Resistance	Phage Type
14	1	+	42D/73 w
	2	+	42D
	3	-	42D/73 w
	4	-	42D/73 w
	5	-	42D/73 w
	6	+	42D/73 w
15	1-6	All resistant	All 42D/73 w
17	1-6	All resistant	All 42D/73 w
20	1	-	42D +
	2	-	42D/54/73/77 42E w +
	3	-	42D/54/73/77 42E w +
	4	+	29/70/42D w
	5	-	42E/54/73/77 w 42D w +
22	1-2	Both resistant	Both 42D/77 w
23	1	+	42D
	2	+	42D/77 w
	3	+	29/52A/50/6/42E 47/54/73/75 +

In column headed phage type) + = additional minor reactions

w = weak reaction

Table 20 Biochemical Reactions and Penicillin Resistance of *Staph. aureus* isolated from "Certified" Milk

Culture No.	Mannitol	Gelatin	Degree of Resistance
3	A	+ slow	R to 5 U
4	A	+ "	R to 10 U
5	A	+ "	R to 10 U
6	A	+ "	R to 5 U
7	A	+ "	R to 10 U
8	A	+ "	R to 5 U
9	A	+ "	R to 10 U
10	A	+ "	R to 5 U
11	A	+ "	R to 5 U
12	A slow	+	R to 5 U
13	A "	+	R to 2.5 U
14	A	+	R to 2.9 U
15	A	+	R to 10 U
16	A	+	R to 1.28 U
17	A	+	R to 5 U
18	A	+	R to 0.64 U
19	A	+	R to 0.64 U
20	A	+	R to 1.28 U
21	A	+	R to 1.28 U
22	A	+	R to 0.32 U
23	A	-	R to 0.32 U
37	A	+	R to 0.32 U
38	A	+	R to 10.3 U
39	A	+	R to 10.3 U
40	A	+	R to 1.28 U
41	A	+	R to 2.57 U
45	A	+	R to 2.57 U
46	A	+	R to 2.57 U
47	A	+	R to 1.28 U
48	A	+	R to 0.8 U
50	A	+	R to 1.28 U

A = acid + = liquefaction R = resistant U = International Units

cultures failing to do so.

The degree of resistance to penicillin shown by the cultures is not outstanding, the highest found being resistant to 10.3 U but not to 20.6 U. Phage typing of the cultures showed them to be mainly 42D some giving many cross-reactions. Sometimes more than one phage type was isolated from the same sample (samples 14 and 20). But in only one case did the phage type of a penicillin-sensitive culture differ from that of a penicillin-resistant one (Nos. 3 and 4 of sample 20).

Discussion of the Observations on staphylococci in "Certified" milk

From the figures given in the preceding tables it can be seen that at least half of the certified milk samples examined during a winter and spring period contained coagulase-positive staphylococci (cf. Tee, 1957). The majority of these staphylococci were found to be penicillin-resistant. There have been other reports of penicillin-resistant Staph. aureus in milk (Price et al., 1954). These workers found penicillin-resistant organisms in cows suffering from mastitis which had never received antibiotic treatment. The organisms were found to belong to phage type 42D, a type not recognised as being pathogenic for man. Other workers have shown that strains of Staph. aureus isolated from normal milk, and from milk of cows suffering from mastitis, mostly belonged to phage type 42D. (MacDonald, 1946, Williams Smith, 1948). The phage types of the organisms isolated during the course of this work confirm this. The majority were of 42D type. Many however, gave varied cross reactions with a wide range of phages. As the phages used in typing were from strains of human origin, it may be that these bovine Staph. aureus cultures should be tested with a new set of phages derived from bovine strains. This is also the conclusion come to by others (Price et al., 1954). For this reason phage typing and penicillin /

penicillin resistance were discontinued. None of the cultures isolated from "Certified" milk belonged to phage types known to be pathogenic for man. Biochemical tests confirmed the view that fermentation of mannitol and liquefaction of gelatin are usually associated with coagulase-positive cultures.

2. Tuberculin Tested Milk

Exactly the same procedure was followed as with "Certified" milk.

Table 21 (page 94) shows nos. of milks examined and nos. of coagulase-positive Staph. aureus isolated during winter and spring periods.

None of the pasteurised samples showed any Staph. aureus. In the winter period 6 of 7 (85.7%) unpasteurised samples showed the presence of coagulase-positive Staph. aureus and 4 of 6 (66.6%) contained penicillin-resistant organisms. In contrast during a spring period only half of the samples, 10 of 21 (47.6%), all unpasteurised, showed Staph. aureus and of these only 3 of 10 (30%) had penicillin-resistant organisms. This may be due, however, to the small numbers examined.

Again the phage types available (table 22, page 95) showed that 42D is common, and there are many cross reactions. In sample 36, the sensitive strain is also type 42Dw corresponding to one of the resistant strains.

Table 23 (page 96) shows the biochemical reactions and penicillin resistance of some of the organisms isolated. All coagulase-positive Staph. aureus fermented mannitol with exception of 1 culture. Not all liquefied gelatin, 4 of 18 cultures failing to do so. The highest degree of penicillin-resistance was again 10.3 U.U. The phage types of cultures again gave many cross reactions and were mainly 42D.

3. /

TABLE 21

Coagulase-positive Staph.aureus isolated from Tuberculin tested Milk

WINTER			SPRING		
Sample	Number of colonies of coagulase + S.aureus isolated	Numbers penicillin resistant	Sample	Number of colonies of coagulase + S.aureus isolated	Numbers penicillin resistant
25P	0	...	1A	3	0
26P	0	...	1B	4	0
27P	0	...	1C	4	3
28P	0	...	1D	0	...
29P	0	...	1E	1	0
30P	0	...	1F	0	...
31P	0	...	1G	2	0
32P	0	...	1H	2	0
33P	0	...	1I	2	1
34P	0	...	1J	1	0
35	6	6	1K	1	0
36	4	3	1L	0	...
37	3	0	1M	0	...
38	0	...	1N	0	...
39	1	1	1O	0	...
40	2	2	1P	2	1
41	1	0	1Q	0	...
42P	0	...	1R	0	...
43P	0	...	1S	0	...
44P	0	...	1T	0	...
45P	0	...	1U	0	...
21	6 (Samples)	4	21	10 Samples	3
%	85.7 (of Samples tested)	66.6 of positive Samples	%	47.6 of Samples	30 of positive Samples

P = pasteurised T.T.
... = no relevant observation.

TABLE 22

Phage Types of Staph.aureus isolated from Tuberculin-tested Milk

Sample No.	Coagulase + S.aureus Colonies No.	Penicillin Resistant	Phage Type
35	1-6	All resistant	All 29w/53w/77w
36	1	+	42D/42Ew/73w
	2	-	42Dw
	3	+	6/7/42E/47/54/73/42Dw
	4	+	6/7/42E/47/54/73/42Dw
37	1	-	29/52/52A/71/53/73/55w 42Ew/54w/3Aw/70w
	2	-	29/52/52A/71/53/73/55w 42Ew/54w/3Aw/70w
	3	-	29w

No more phage types available

w = weak reaction (in phage type column).

TABLE 23

Biochemical Reactions and Penicillin Resistance of *Staph. aureus* isolated from Tuberculin Tested Milk

Culture Lab. No.)	Mannitol	Gelatin	Degree of Penicillin Resistance
24	A	+	R to 0.16 I.U.
25	A	+	R to 0.32
26	A	+	R to 0.32
27	A	+	R to 0.32
28	A	+	R to 2.57
29	A	+	R to 2.57
30	A	+	R to 2.57
31	A	-	R to 2.57
32	A	-	R to 1.28
33	A	+	R to 0.08
34	A	+	R to 10.3
35	A	+	R to 0.64
36	A	+	R to 10.3
42	A	-	R to 0.64
43	A	+	R to 0.32
44	A	-	R to 0.32
49	A	+	R to 0.16
52	-	+	R to 0.16

A = acid

\+ = liquefaction

\- = no reaction

R = resistant

I.U. = International unit.

3. Pasteurised Milk

None of the samples examined showed the presence of coagulase-positive staphylococci, as would be expected if pasteurisation is efficient.

4. Staphylococci from mastitis milks

57 cultures of staphylococci were received from the West of Scotland College of Agriculture. Of the 50 coagulase-positive organisms, 20 were chosen for further study. These were isolated during routine examination of samples from individual quarters of the udders of cows suspected of having mastitis. Only coagulase-positive cultures were investigated and they were tested for penicillin resistance, biochemical reactions, and phage type. Results are shown in table 24 (page 98).

All cultures fermented mannitol and liquefied gelatin, and the highest degree of penicillin resistance was 10.3 U. Again phage types gave many cross-reactions and were mainly 42D. There were no differences between the phage types of penicillin-resistant and penicillin-sensitive cultures. The phage types did not differ from types obtained from normal milk samples, which would indicate that phage typing with the phages available would not be a good method for determining if Staph. aureus was the causal organism in doubtful cases of mastitis.

Discussion

During the examination of milks of different grades in winter and spring 1956-57, a total of 110 cultures of coagulase-positive Staph. aureus were isolated, and many of these were, in addition, penicillin-resistant. More than half (winter 54%, spring 59%) the samples examined contained Staph. aureus, and of these more than half (winter 61%, spring 55.5%) were penicillin-resistant. This is in agreement with Tee (1957), who found that 80% /

TABLE 24

Biochemical Reactions, Penicillin Resistance, and Phage Type of Staph. aureus isolated from Mastitis Milk

Culture	Biochemical Reactions: Mannitol	Biochemical Reactions: Gelatin	Degree of Penicillin Resistance	Phage Type
A	A	+	R to 0.64	29/42E/73/47w +
E	A	+	R to 0.64	29/42E/73/47/6w +
6	A	+	R to 0.32	75
F	A	+	R to 0.64	29/42E/47/52A/54/79/6/73w +
G	A	+	R to 0.64	29/42E/47/52A/54w/73w
T	A	+ slow	R to 1.45	42Dw
W	A	+	Sensitive	29/52Aw +
B	not examined		"	52A/55/42Ew/54w/73w
C			"	29/52A/6/42E/47
H			"	42Dw
I			"	
IG			"	42E/54/73/42D
L			"	42E/54/73/42D
2L	A	+	"	42E/54/73/42D
M	not examined		"	42E/54/73/42D
P				42E/54/73/42D
2E	A	+ slow	R to 2.57	6/7/42E/47/54/73/75
2H	A	+	R to 5.15	6/7/42E/47/54/73/75
2K	A	+	R to 2.57	N.T.
2N	A	+	R to 2.57	77

N.T. = not typable
In phage column + = additional reactions present
In gelatin column + = liquefaction
A = acid

80% of herd samples had Staph. aureus, and MacDonald (1946) who found that 35% accredited milks had Staph. aureus. On further investigation the cultures gave typical biochemical reactions of Staph. aureus (fermented mannitol 99.1%, liquefied gelatin 95.5%). The cultures were examined for phage type and degree of penicillin resistance.

Phage typing revealed that none of the organisms were of types known to be pathogenic for man and many belong to group 42D which is known to be commonly found in milk. The phage types were very complex. A series of cultures of Staph. aureus obtained from mastitis milks, gave similar results. It would therefore seem that:-

(1) The cultures isolated are not of types known to be pathogenic for man.
(2) There can be no distinction between organisms from normal and mastitis milks by phage typing with the phages available.
(3) There is no significant relationship between phage types of penicillin-resistant and penicillin-sensitive organisms.
(4) The penicillin-resistance of cultures was not exceptional (the highest was to 10 I.U.).
(5) Large numbers of Staph. aureus, although apparently harmless in practice, are not desirable in consumer milk.
(6) A range of phages propagated on bovine strains of Staph. aureus might be more suitable for typing organisms from milk.

Summary

Staph. aureus was isolated from consumer milks of different grades and many of the organisms were penicillin-resistant. On phage typing, they were found to be mainly 42D, a type common in milk. A series of cultures from mastitis milks showed no significant differences from those isolated from milk of normal cows, as far as
is /

is known.

As the phage types of Staph. aureus isolated from consumer milks are not known to be pathogenic for man, the practical significance of their appearance is not as great as it might seem and has often been rather uncritically held to be.

SECTION III

Correlation of Resazurin Test and Plate Count at 10-15°C, with the Presumptive Coliform Test

Procedure for the Resazurin test is described in "Methods". The survey was carried out from January to October, 1959.

Results

1) Pasteurised Milk

Table 25 (page 102) shows the failures in the Resazurin test, psychrophile count, and coliform test (at standard dilutions) for the year 1959, and for the different seasons of the year.

None of the samples examined failed the Resazurin test. There were no coliform failures in winter; all but one were found in the warmer summer months. Similarly, the greatest number of samples failing the psychrophile count were discovered in summer. The coliform failures were of the same order as those found in previous years. Interesting points arise when the three tests are correlated. The coliform failures were always correlated with a failure in the psychrophile count, yet these samples passed the Resazurin test. As already mentioned, Thomas et al., (1949) suggested that psychrophile counts were valuable in detecting post-pasteurisation contamination. From the results obtained here, it would seem that the coliform test, which is easier to perform and gives quicker results, is just as efficient.

The different types of coliforms isolated from the presumptive tests are shown in table 26 (page 102).

As in previous years the chief coliform contaminants in pasteurised milk appear to be K.aerogenes and intermediate coliforms.

2) /

Table 25 - Failures in Resazurin Test, Psychrophile Count and Coliform Test for Pasteurised Milk

	Total Examined	Failing Standard Coliform	Psychrophile count over 10,000/ml.	Failing 1 hr. Resaz.	Failing Coli & Resaz.	Failing Psych.& Resaz.	Failing Psych. & Coli.
Whole Year	47	10	28	0	0	0	10
Winter	15	0	8	0	0	0	0
Spring	10	1	3	0	0	0	1
Summer	22	9	17	0	0	0	9

Table 26 - Types of Coliforms Isolated in Positive Coliform Tests for Pasteurised Milk

Total isolated	E.coli	K.aerogenes	Intermediate Coliform	Others
10	1	3	6	0

2) "Certified" Milk

Table 27 (page 104) shows the failures in the Resazurin test, psychrophile count, and coliform tests for "Certified" Milk.

Here there are four samples failing the Resazurin test and all were discovered during the summer months. The four samples failing the Resazurin test also failed the psychrophile count and coliform test (and plate count at 37°C), i.e. they were extremely bad failures. As with pasteurised milk, there was a close correlation between failures in the coliform test and psychrophile count. Again there was a higher failure in coliform tests and psychrophile counts in summer than in winter, as would be expected. The different types of coliforms isolated are shown in Table 28 (page 104).

Here the predominating organisms are E.coli and intermediate coliforms. In two of the tests, no coliform organisms or other significant organisms were isolated.

The results obtained for pasteurised and "Certified" milk would appear to indicate that the Resazurin test is far from being a suitable test for grading milk. It will detect a very bad milk sample, but many of the samples which passed the Resazurin test failed the coliform test in very high dilutions, and had completely uncountable plates for the psychrophile count and for the count at 37°C (Table 29 page 104).

Although only the results of the 1 hour Resazurin test are recorded in the tables, the test was incubated for a further 5 hours and none of the samples passing the test would have failed it after 2 hours, which is the maximum time laid down in the Scottish Milk Testing Scheme for the Temperature-Compensated Resazurin Test. The test may be of value at creameries where many supplies /

Table 27 - Failures in Resazurin Test, Psychrophile Count, and Coliform Test for "Certified" Milk

	Total Examined	Failing Stand. Coli.	Psychrophile Count over 10,000/ml.	Failing 1 hr. Resaz.	Failing Coli & Resaz.	Failing Psych.& Resaz.	Failing Psych.& Coli.
Whole Year	65	28	45	4	4	4	21
Winter	22	4	12	0	0	0	2
Spring	18	7	13	0	0	0	5
Summer	25	17	20	4	4	4	15

Table 28 - Types of Coliforms Isolated in Positive Coliform Tests for "Certified" Milk

Total Examined	E.coli	K.aerogenes	Intermediate Coliform	Others
28	13	3	10	2

Table 29 - Level of Dilution at which Coliform Tests Failed for "Certified" Milk

	Dilutions of Milk		
Total Failures	1/10	1/100	1/1,000
28	7	6	15

supplies are bulked and where inclusion of one very bad milk may spoil the whole collection. But the daily test employed in this case is only a 10 minute Resazurin test which is capable of detecting only extremely unsuitable milks.

Of the three tests which have been compared, I think that the coliform test is still the most suitable for both pasteurised and "Certified" milk. The Resazurin test is not dependable and detects only very poor milks. The psychrophile count, although useful in detecting post-pasteurisation contamination, is cumbersome and suffers from the same errors as the plate count at 37°C, with the added inconvenience of a much longer incubation period. The coliform test will fail milks which have a high psychrophile count and also milks which pass the Resazurin test, but which contain E.coli.

DISCUSSION

The object of this work was to collect informatioon on the bacterial flora of milk as it reaches the consumer, with emphasis on the incidence of positive presumptive coliform tests in such milks. It was hoped that such information might settle some of the arguments for and against discontinuing the test in Scotland.

It has been suggested that the coliform test should be abandoned for the following reasons:-

1) It does not have the same significance in milk as in water. The presence of coliform organisms or even of E.coli type I in milk does not necessarily prove direct faecal contamination.

2) There is a high error in the dilution method, and the organisms finally isolated by differential means may give no indication of the true coliform population which was present originally.

3) The test is selective for coliform organisms - other more harmful pathogenic bacteria will not be detected.

4) Cross bacterial contamination of milk may occur in the absence of coliforms (Newman, 1951).

5) Milk is a good medium for bacterial growth and unless it has been kept at a temperature of 40°F or less, a great increase in coliform population may occur, and an estimation of coliform organisms may give a misleading index of the original contamination.

In answer to these objections, several points may be advanced in favour of retaining the test.

1) It was not intended as a test for faecal contamination, but as an index of careless methods of production, sterilisation of milking utensils, and insufficient cooling of milk after production.

2) Although the presence of E.coli type I does not necessarily make milk unfit for human consumption, there is the possibility that some of these organisms may be of the serotypes responsible for infantile gastro- /

gastro-enteritis in young children. The fact that the same organisms are responsible for white scour in calves, makes the possibility of their presence in milk greater than might be expected.

3) Insignificant initial contamination with a few coliform organisms may be of no importance, but if milk is not properly cooled they will increase in numbers as will most other bacteria present. Therefore the presence of large numbers of coliforms is significant whether it is due to initial gross contamination, which should be avoided, or whether it is due to multiplication of the organisms because of insufficient cooling of the milk after production.

4) It has been suggested that by choosing coliform organisms as an index of unclean milk, we have selected a type of organism whose presence in milk has no real significance. The coliform test will not detect the presence of other pathogens in milk. There is, however, no simple test which will detect all the possible pathogens present in milk. A special test would have to be devised for each one, and this would only add to the complications of milk testing.

5) In view of the results obtained in this work, it would seem that the coliform test should be retained. Milk when it reaches the consumer is far from being free from coliforms. This is especially true of "Certified" milk. In addition, 57.2% of the coliforms isolated from "Certified" milk are E.coli. These results are similar to those obtained by Brodie (personal communication) whose survey showed that 40.6% of positive tests were due to E.coli. The high incidence of coliforms in consumer milk suggests that the test should not be relaxed because the presence of even a few organisms may lead to a considerable population of coliforms by the time the milk reaches the consumer. Indeed it is doubtful if outgoing supplies are tested regularly, (Report on Milk Services, Scotland, 1949).

6) The question of keeping quality is also involved. Coliforms present /

present in large numbers in milk will reduce its keeping quality and if the milk is to be used for cheese manufacture, they may affect the flavour of the cheese.

If the coliform test is abandoned, what other test can take its place? And are we justified in selecting this test for removal when others are retained which might reasonably be regarded as less useful? It may be argued that a total count of the bacterial population is a better indication of the hygienic quality of milk (Newman, 1951). That the plate count is subject to many errors is well known (Wilson et al., 1935). Apart from errors of dilution and errors due to clumping of organisms, many of the organisms may not grow at the temperature of 37°C laid down in the standard methods. McKenzie (1952) is in favour of incubating the colony count at 30°C on the basis of the fact that many organisms present in milk have a much lower optimum temperature than 37°C. This involves a period of incubation of 72 hours instead of 48 hours and the other errors are still not eliminated.

Now that a greater proportion of milk is being collected by the bulk tanker method, it has been suggested that a psychrophile count would give a much better indication of poorer methods of production in this type of milk. Here again the disadvantage lies in the much longer incubation period required. For pasteurised milk psychrophile counts give a good indication of post-pasteurisation contamination (Thomas et al., 1949). In the present work they have correlated completely with the coliform test. High psychrophile counts occurred in milks failing the coliform test.

It seems to me that my results indicate that the coliform test should not be abandoned, as it seems to give more consistent results than many of the other tests, and it is a good indicator of keeping quality and hygienic methods of production. It should be retained /

retained for both "Certified" and Pasteurised grades of milk and the standard might be raised in the case of Pasteurised milk from "absent in 0.01 ml." to "absent in 0.1 ml."

Results show that Pasteurised milk is very variable in regard to passing the coliform test. As would be expected there is a higher percentage of failures during spring and summer. Samples which had coliforms present in 0.01 ml. also had coliforms in 0.1 ml. and there were very few samples which had coliforms in 0.1 ml. and none in 0.01 ml. It would seem therefore, that raising the standard to "absent in 0.1 ml." would still fail the milks showing coliforms in 0.01 ml. and would increase the overall failures by very little. Pasteurised milk is supposed to be heat-treated to destroy any potentially pathogenic organisms which may be present. Coliform organisms should also be destroyed if the heat treatment has been efficient. The fact that pasteurised milk often gives a positive coliform test in fairly high dilutions, suggests a source of post-pasteurisation contamination. The source most often responsible is the insufficiently cleaned milk bottle (Hobbs & Wilson, 1943). There would seem to be little point in pasteurising milk only to put it into unclean bottles, thereby undoing all the careful treatment which has gone before. For this reason pasteurised milk should have a fairly high standard and should not contain large numbers of coliforms. That post-pasteurisation contamination is caused most often by K.aerogenes and intermediate coliforms is probably an indication that contamination is from improperly washed bottles and equipment.

With pasteurised milk there were 8 of 137 positive tests from which coliforms were not isolated. This is a much higher incidence of false positives than was obtained with "Certified" milk, where only 1 of 135 was not caused by coliforms. Only one of these false /

false reactions occurred in the dilutions set by the present standard and would have failed the 1 in ten or 1 in a hundred dilution. In addition it would have failed on the plate count.

"Certified" milk has a large number of failures at all seasons of the year, and they are exceptionally high in summer, in fact, approximately 50% of samples examined failed the test. The coliform most often isolated from "Certified" milk was E.coli, suggesting that contamination was more frequent and more likely to be due to sources other than improperly washed bottles. False positive tests were almost absent in this grade of milk. "Certified" milk is not heat-treated in any way, and it is therefore desirable that it should be kept as free from bacterial contamination as possible. The high incidence of positive coliform tests, and the fact that half of them are due to E.coli is an indication that the milk is not being produced under hygienic conditions.

The milk samples examined in this survey were of unknown age. It may be argued, therefore, that the results are not valid because the total bacterial and coliform populations will have increased considerably since the time of production (Clegg et al., 1949). The fact remains that these samples which the consumer receives are certainly not of the best bacterial standard, therefore every attempt must be made to ensure that the milk which leaves the producer is of the highest standard because the delays involved before it reaches the consumer will cause rapid deterioration of even the "best" milk. These results seem to be typical of the standard of Scottish milk. Brodie (personal communication) has made a statistically larger survey of milk (1,200 samples of different grades) and found that 26.3% of samples failed the test at standard dilutions and of these failures, 40.6% were due to E.coli type I faecal variety.

With /

With the evidence of these results in mind, it would surely be a retrograde step to abandon a test, which, if applied more conscientiously to sampling, would prevent further deterioration in the standard of Scottish milk.

If the coliform test were to be abandoned, we should have little idea of the extent of contamination of the milk. It is understandable that it is not easy to produce milk completely free from coliform organisms, especially if the producer has very few modern appliances at his disposal. Evidence that conditions are not quite what they should be on farms producing the "Certified" grade of milk is shown by a newspaper cutting concerning the dairy herd at Boquhan Farm, Kippen.

Copy of newspaper article ("Evening Citizen", 11th December, 1957).

"AYRSHIRES 'KNOCKED OUT' BY MUD

"The pedigree dairy herd owned by 46-year old Mr. John Pirie, Boquhan Farm, Kippen, Stirlingshire, comes under the auctioneer's hammer to-day - because of muddy fields.

"Mr. Pirie started pedigree cattle breeding 25 years ago when he was 21.

"At that time he was working on 'dry' land at Lennoxtown. But ten years ago he moved to Boquhan Farm, and found himself in difficulties with the heavy type of ground.

"So to-day his 180-strong herd of Ayrshire cattle and dairying equipment - including delivery vans - are being sold off on the farm to make way for a new beef herd.

"'SUITS BEEF'

"'The land is heavy and animals coming in and out of the /

the fields for milking got into a terrible mess in wet weather. The clay stuck to them', said Mr. Pirie yesterday.

"'The land is more suitable for beef and cropping. It is my own decision, and a lot of people think I am making a mistake.

"We produced 200 gallons of certified milk a day, supplying some to shops, retailing the rest. Everything was bottled on the farm. Already the first beef animals are on the farm - a small herd of 11 shorthorns.' In spring Mr. Pirie will start adding to them."

23 of the samples which I examined came from this particular source, and 19 of these (82.6%) had positive coliform tests. 10 of these were caused by E.coli. The conditions on this farm were obviously unsuitable for the production of milk of the "Certified" grade. Thus, the coliform test ought to be retained as a useful check on the production of milk which will not be pasteurised.

If the coliform test is abolished what tests will be substituted for it? The plate count at 37°C is already a standard test but is subject to an error of ± 64% (Chalmers, 1955), and does not indicate the type of organisms present. The plate counts carried out on the milk samples I examined agreed very closely with the coliform test. For "Certified" milk, failure on the plate count was accompanied by failure in the coliform test in every case except two. Of these two samples, one had a negative coliform test and the other contained coliforms but not in the dilutions which would fail it. Although there is no standard plate count for pasteurised milk, many of these samples had counts well above the "Certified" standard. Again, little reliance can be placed on these results because post-pasteurisation contamination may alter the picture considerably, and /

and in addition heat-treatment may select a population of organisms which will multiply rapidly in insufficiently cooled milk to give a high plate count.

Examination of milk for the presence of Cl. welchii showed that the organism was present in both pasteurised and "Certified" milk. A test for the presence of Cl. welchii, however, is not one which might be applied to detect contamination of milk, because the organism will be found in many sources on the farm, and could gain access to milk even if there had been no faecal contamination. Just as false-positive coliform tests may be produced by organisms other than coliforms, it was found that "stormy clots" may be caused by organisms other than Cl. welchii.

The fact remains that most of the false-positive coliform tests encountered were found only in the tubes containing 1ml. of undiluted milk. This led to a series of experiments which showed that addition of 1ml. of milk to MacConkey medium greatly enriches it, thereby allowing organisms which might be inhibited by bile salt, to multiply. Many of the streptococci normally present in milk will grow in MacConkey broth to give an acid reaction. Other proteolytic organisms, e.g. Proteus vulgaris, Bacillus subtilis, Cl. welchii, and Cl. sporogenes, will grow in the presence of 1ml. of milk. The milk provides a protein substrate for these organisms.

This is a possible explanation of the false-positive reactions. As they occur almost exclusively in the tubes which would be ignored by the standard dilution tests, the argument that the coliform test should be abandoned because very often positive tests are not due to coliforms, has little value. I have found consistently that tests failing the standard dilutions have always contained coliforms.

An attempt to find a substitute for the coliform test led

to /

to the examination of milk samples by the plate count at lower temperatures (10-15°C), because the organisms most likely to be present in milk would have optimum temperatures in this region. I found that a high psychrophile count correlated more often with a positive coliform test than did the plate count at 37°C. There were many instances, however, where high psychrophilic counts were accompanied by negative coliform tests. It would seem from these results that plate counts at a lower temperature give a better idea of the bacteriological quality of milk than counts at 37°C.

Unfortunately the longer time required for incubation makes this test cumbersome to perform and the results are not known for several days. On the whole, the coliform test appears to be the quickest and easiest and most reliable test for the detection of faulty and unclean methods of production. It correlates very closely with the psychrophile count which is a good indication of the hygienic quality of milk, but which takes more time and materials to perform. A suggested modification of the test (Brodie, 1959) is to incubate the MacConkey tubes at 37°C and also at 44°C. The latter test gives a direct indication of the presence of E.coli type I and the former a general indication of the total coliform contamination.

The Resazurin test is much in favour at the moment, and it is suggested that it should replace the coliform test in a new modified form (Morgan, 1959). From the results obtained in this work, it would seem that the Resazurin test fails only the very poor milks and does not detect those which may rapidly become of poor bacteriological quality shortly after testing. Under the present Scottish Milk Testing Scheme, daily 10-minute Resazurin tests and a weekly test for keeping quality by the Temperature-Compensated Resazurin test, are carried out on milk which is to be bulked. In view of the fact that /

that the Resazurin test detects only extremely poor milk, any move to make this the test on which most milks are passed or failed must lead to a deterioration in the standard of milk supplies.

There is another dye-reduction test, the Methylene Blue test, which is carried out on milks which fail the weekly Resazurin test. It is generally agreed (Wilson, 1935, Thornton & Hastings, 1930) that this test is a good measure of the keeping quality of milk. It would seem to be a more reliable test than the Resazurin test, and may detect mastitis milk which reduces methylene blue much more quickly than normal milk (Nilsson, 1959). This is not due to a large bacterial population because many of these milks contain very few organisms. It has been found that in mastitis milk the donors or precursors for the xanthine oxidase enzyme system are present in fairly high concentration which will result in a fall in potential at 37°C and will decolourise methylene blue, (Nilsson, 1959). The rate of oxidation of the adenine-flavine-dinucleotide prosthetic group of xanthine oxidase is very slow but the addition of the reversible dye methylene blue, which acts as a carrier between the flavoprotein and oxygen, allows quicker oxidation of the flavoprotein and enables the reduction of methylene blue to progress more quickly.

The Resazurin dye-reduction test was compared with plate counts at the two temperatures (10°-15°, and 37°C) and also with the coliform test. The results of these experiments showed little relationship between dye reduction and bacteriological content. Practically all milk samples passed the 1 hour Resazurin test but failed either the plate counts, or the coliform test, or both.

Anderson and Wilson (1945), examined 2,588 samples of milk by the 10 minute Resazurin test, the one hour Resazurin test, the Methylene Blue test, and keeping quality tests. They found that only 53% of milks failing the 10 minute Resazurin test reduced methylene blue /

blue within 30 minutes, (a rejection test, because such milk has a keeping quality of only 5 hours). The authors concluded that the Methylene Blue test is the best indirect index of keeping quality. A less accurate test is the 1 hour Resazurin test, but the 10 minute Resazurin test will pass nearly 50% of unsatisfactory milks.

The results obtained in this survey indicate that of all the tests employed in the bacteriological control of milk supplies, the coliform test is the most reliable. False positive results are not as common as one is led to believe, and seldom occur outside tubes receiving 1ml. of milk. The test is of value in controlling hygienic methods of production and in ensuring milk of good keeping quality. I think that the coliform test gives the best indication of these standards and should not be abandoned as a routine test.

The Resazurin test would seem to detect only very poor milks and is not therefore suitable for standardising the general milk supply. The Methylene Blue test would appear to be more reliable for this purpose. It has been found that there is a relationship between the reduction time in this test and the coliform content of the same sample (Nilsson, 1959). Thus these two tests seem to offer the best solution to the bacteriological grading of milk.

SUMMARY

1) A survey was made of the bacterial flora of consumer milk in Glasgow for the years 1956-59, with special emphasis on the presumptive coliform test.

2) Both pasteurised milk and non-pasteurised milk showed a high proportion of positive coliform tests.

3) Positive tests were more common in summer than in winter.

4) Of 171 positive coliform tests, only 9 were caused by organisms other than coliforms, and of these, only 1 was positive in dilutions which would have failed the standards laid down by the authorities.

5) The incidence of different types of coliforms varied for pasteurised and "Certified" milk. K.aerogenes and intermediate coliforms were isolated most often from pasteurised milk, whereas in "Certified" milk E.coli was the type most frequently isolated.

6) Organisms responsible for false positive coliform tests included Cl. welchii associated with a Pseudomonas sp., Proteus vulgaris associated with a streptococcus, and aerobic sporing bacilli. False positives seem to be enhanced by the presence of 1ml. of undiluted milk in the MacConkey medium. It is suggested that this counteracts the selective action of the bile salt.

7) Plate counts at 37°C and at 10-15°C are not reliable indicators of the bacteriological standard of milk.

8) Cl. welchii can be isolated from both pasteurised and non-pasteurised milk. "Stormy clots" can very often be produced by organisms acting synergistically, and may not be caused by Cl. welchii. No non-haemolytic food-poisoning strains were isolated from the milks examined.

9) Unpasteurised milk, ("Certified") very often contained large numbers of coagulase-positive Staphylococcus aureus. Phage typing revealed that the majority of these belonged to group 42D, which is common /

common in milk. About half of the strains isolated were penicillin-resistant. It is suggested that the higher incidence of staphylococcal mastitis is due to the indiscriminate use of penicillin. Although the strains isolated do not belong to phage types known to be pathogenic for man, it is undesirable to have large numbers of these organisms in milk.

10) The Resazurin test is not a good indication of the bacteriological condition of milk. Practically all the samples examined passed the Resazurin test, but many failed either the plate count or the coliform test or both.

11) In view of the varied and doubtful flora of "Certified" milk, as much milk as possible should be pasteurised. Coliforms and Staph. aureus may have little effect on adults consuming milk, but may cause intestinal upsets in young children.

12) The standards for pasteurised milk should be more exacting, as this is meant to be a "safe" milk free from most contaminating bacteria.

13) The use of tetrazolium salts in media for the quick detection of coliform organisms in milk and water does not seem to be sufficiently selective. Results tend to be inconsistent.

APPENDIX

1) Identification of Intermediate Coliforms giving a '++++' 'I.M.V.iC.' Reaction.

The method employed was that of Møller (1954), already described in "Methods". Altogether four cultures were obtained which gave a '++++' 'I.M.V.iC.' reaction. Three of these cultures, K1, K2, and K3, were obtained from the same milk sample, but differed slightly in their colonial appearance and one had a doubtfully positive Voges-Proskauer reaction. The biochemical reactions are shown in Table 30 (page 120).

To all intents and purposes, these cultures were identical, but fell neither into the genus Escherichia nor the genus Klebsiella. Examination of the amino acid decarboxylases by Møller's method showed that K1, K2, K3, were identical but differed from M. Culture M contained lysine, ornithine, and arginine decarboxylase. K1, K2, and K3 contained lysine and arginine decarboxylase but no ornithine decarboxylase.

Thus it would seem that culture M belongs to the intermediate group I, as its amino acid decarboxylase pattern is similar to that found in the Hafnia genus. Cultures K1, K2, and K3, are similar to the genus Klebsiella.

2) Gram-negative Rods Giving "L" Form-stained Morphology on MacConkey Agar.

On two occasions, when examining Gram-stained films of colonies on MacConkey plates, an unusual morphological appearance was observed. (Plate 6a, b, c, pages 136,137). The organisms were Gram-negative rods, but there were also present long, thin, Gram-negative filaments, some of them containing swollen structures which were granular and stained Gram-positive. They were very similar to the "L" forms of Proteus when it is grown on media containing /

Table 30 Biochemical Reactions of the Intermediate Coliforms M, K1, K2, and K3

Culture	Lact.	Gluc.	Suc.	Man.	Dul.	Mal.	Litmus Milk	I.	M.	V.	C.
M	AG	AG	AG	AG	-	AG	A	+	+	+	+
K1	AG	AG	AG	AG	-	AG	A	+	+	+	+
K2	AG	AG	AG	AG	-	AG	A	+	+	+?	+
K3*	AG	AG	AG	AG	-	AG	A	+	+	+	+

* flatter colonies than K1 and K2.

containing penicillin. When subcultured on to nutrient agar, these forms disappeared, (Plate 10a, 10b) (page 141) suggesting that the bile salt of MacConkey medium may play some part in inducing their formation.

One of these organisms was retained for further study. Biochemical reactions showed it to be E.coli. (Table 31) (page 122).

The organisms were motile, but when a flagellum stain was performed (Kirkpatrick's silver stain), flagellation appeared to be sub-polar, and not peritrichous as befits the members of the Enterobacteriaceae. (Fig. 5) (page 123).

Here we have a rather awkward problem in taxonomy. All the physiological and biochemical properties of this organism point to its being E.coli. Its flagellation, however, is not peritrichous - one of the main requirements for the inclusion of an organism in the family Enterobacteriaceae. Another peculiarity is the production of abnormal morphological forms when the organism is grown on MacConkey agar. The colonies differed from the normal type of E.coli colony (Plate 9a) (page 140) in that they had irregular edges and had a "poached egg" appearance - there being a slightly smooth, raised, part in the centre of the colony. (Plate 9b, 11, pages 140,142).

These morphological forms were constantly produced on MacConkey agar, even when the organism was maintained for some time on nutrient agar. Attempts were made to produce these "L" forms on agar containing penicillin - the usual method of producing them in Proteus, Streptococcus, and Salmonella species. The culture was grown on nutrient agar containing 5, 10, and 800 U. of penicillin. No change in morphology was observed. The organism appeared as a normal Gram-negative rod. "L" forms were not observed.

In order to determine whether the bile salt in MacConkey medium was responsible for the appearance of "L" forms, the organism was /

Table 31 Biochemical Reactions of "L form" (Culture 25)

Lact.	Gluc.	Suc.	Man.	Dul.	Mal.	Lit. Milk	I.	M.	V.	C.	Gel.	H_2S	Urea	Eij.	Mot.
AG	AG	AG	AG	-	AG	A	+	+	-	-	-	-	-	+	+

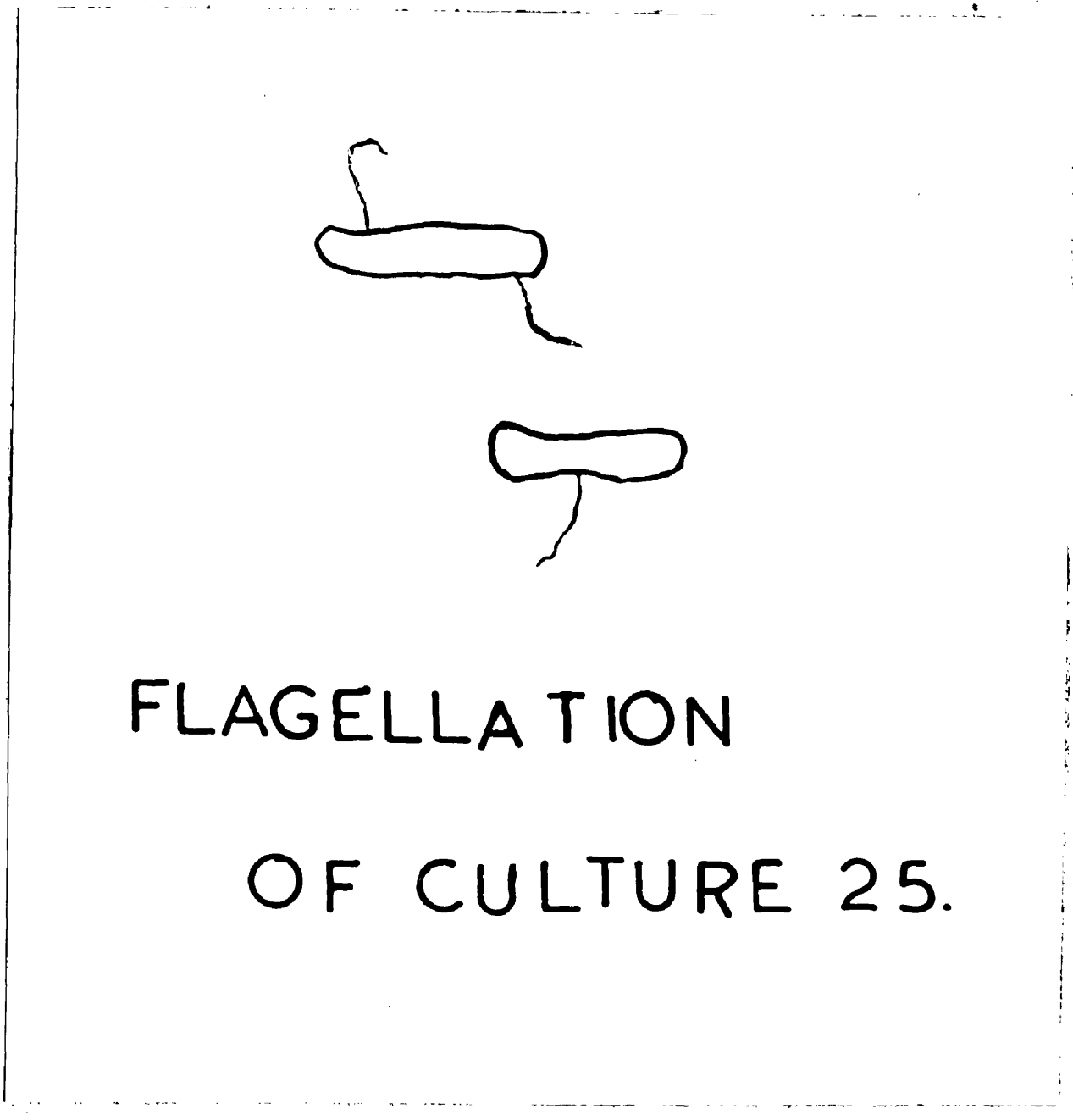


Fig. 5. Flagellation of culture 25.

was grown on MacConkey agar prepared in the normal way but with the omission of bile salt. Again the characteristic bulging filaments were obtained. The only other constituent of MacConkey medium which would be likely to induce such appearances was lactose. Accordingly, nutrient agar plates were prepared containing 1% lactose. On this medium the "L" forms were extremely marked and exaggerated. On plain nutrient agar, the organisms are normal Gram-negative rods, therefore the production of these "L" forms is apparently due to the influence of lactose.

A peculiar globular form of Lactobacillus bifidus has been reported by Sundman and Björksten (1958). This appeared when L.bifidus was grown on tomato agar, and disappeared when tryptic digest of cows' milk was added to the medium. The authors concluded that the globular forms were the response of L.bifidus to a nutritionally deficient medium, because cell wall synthesis was more exacting for this organism, and could not take place on such a deficient medium. Lark (1958) obtained globular forms of Alcaligenes faecalis in the presence of penicillin and he put forward the hypothesis that penicillin inhibits some component in the cell which is concerned with cell division and formation of the cell wall.

It seemed to me that a similar explanation would account for the globular forms of E.coli. Cell wall staining by the tannic acid-crystal violet method revealed that the globular forms had no cell walls, whereas the normal bacillary forms and shorter filaments had.

From these results I would suggest that this particular strain of E.coli differs nutritionally from the normal ones, because when it is grown in the presence of lactose, normal cell wall synthesis is blocked or inhibited in some way.

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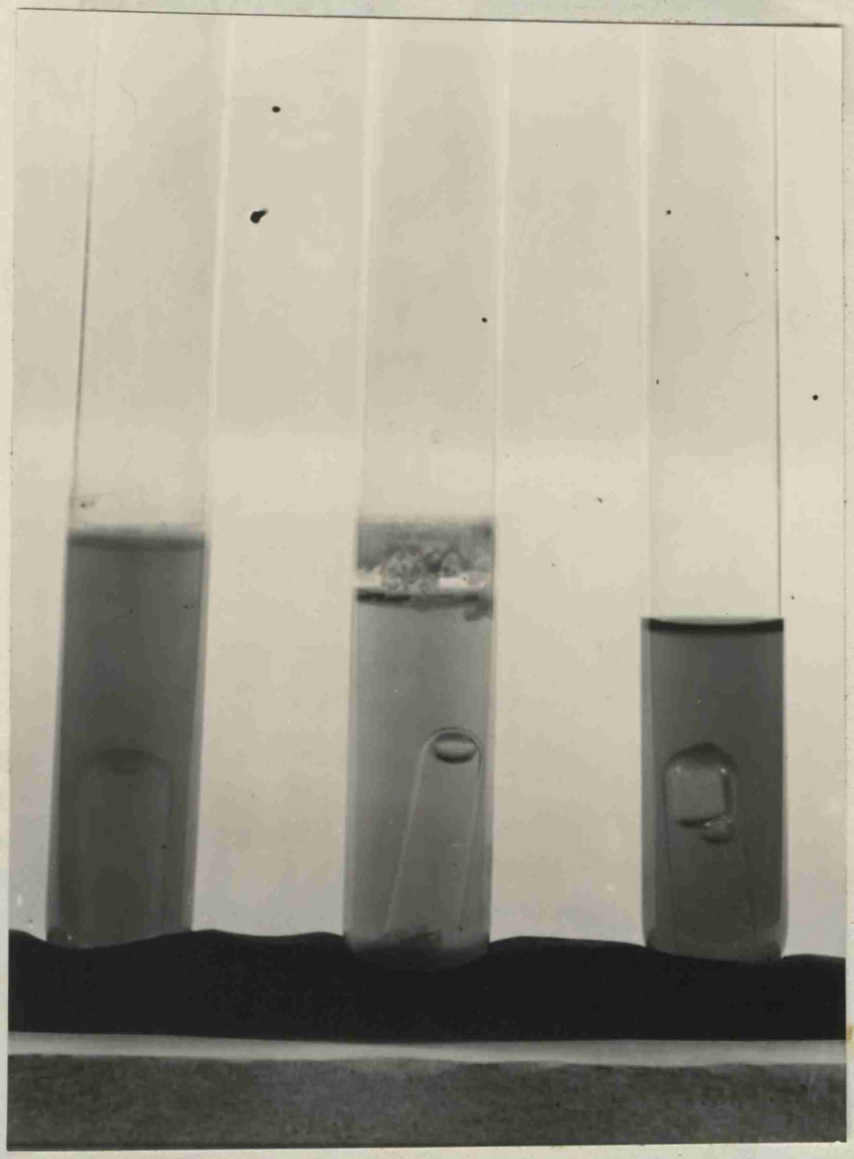

PLATE I. Small gas volumes were often observed in false-positive coliform tests. From left to right:- (1) false positive, (2) false positive, (note fatty scum on surface of medium), (3) E.coli.

PLATE 2A. False-positive presumptive coliform test due to Cl.welchii. Left to right; (1) original; (2) subcultured aerobically, no gas; (3) subcultured anaerobically (small gas volume).

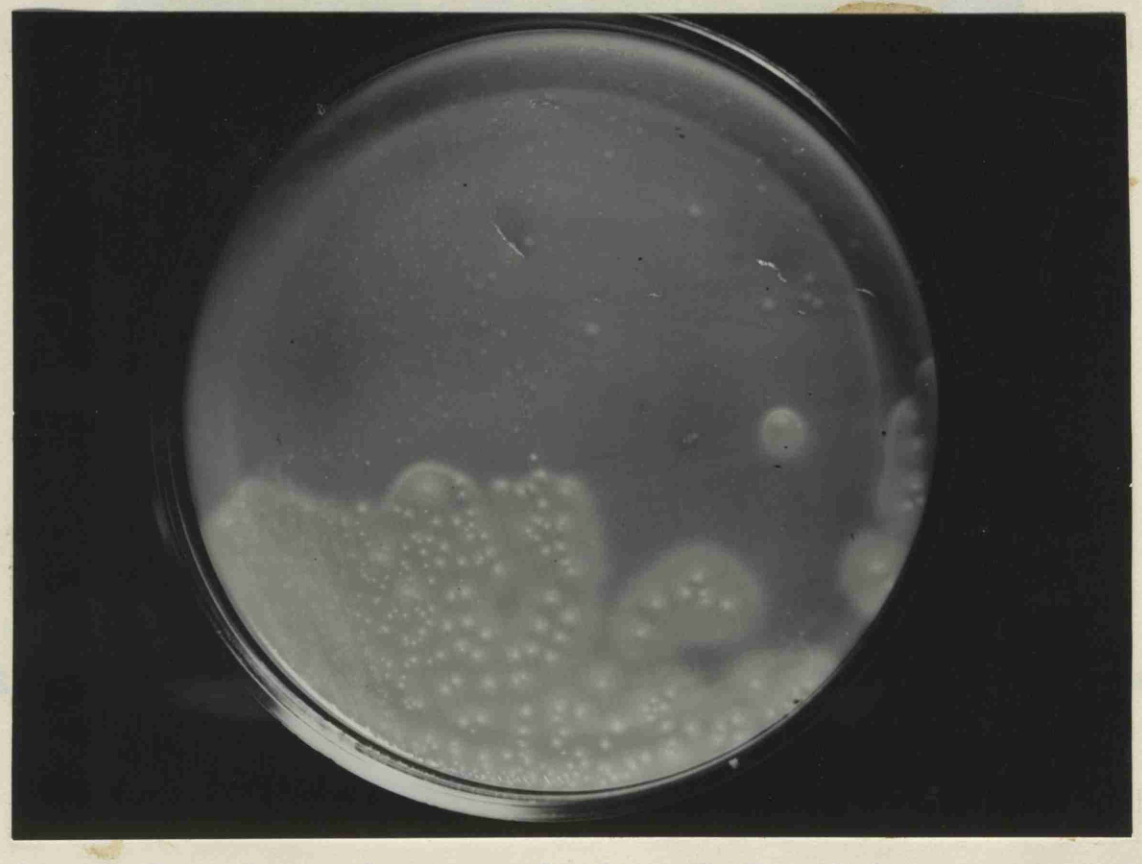

PLATE 2B. Nagler plate showing colonies of Cl.welchii surrounded by a turbid halo on the ~~left~~ LOWER half, which has not received any anti toxin.

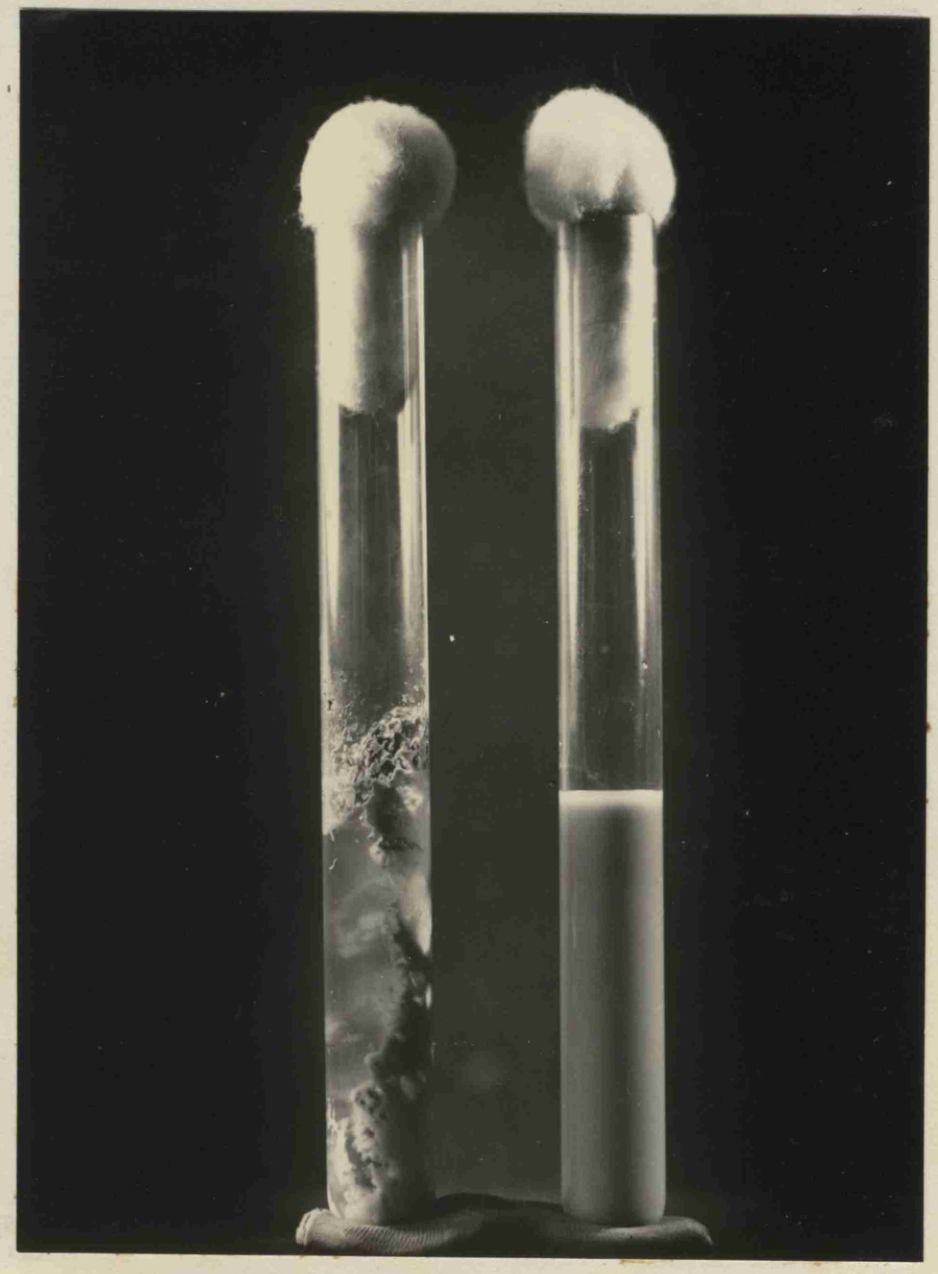

PLATE 3. False "Stormy clot". Only Lactobacilli and micrococci isolated.

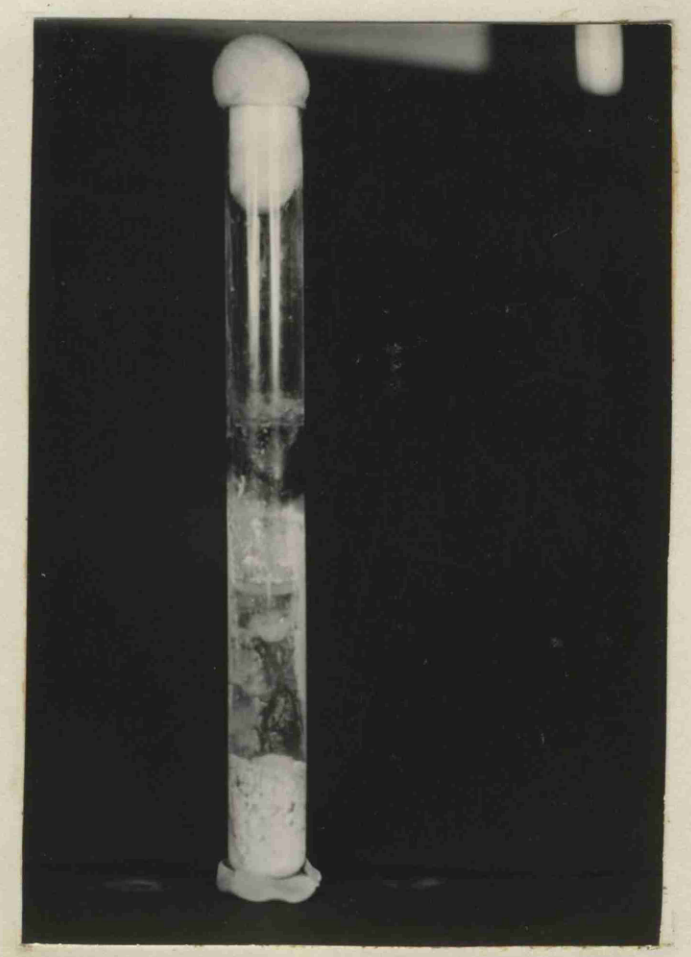

PLATE 4. False "stormy clot". Only B. subtilis isolated.

PLATE 5. False-positive "stormy clots".

(1) left, sample h, E.coli and streptococci isolated.

(2) right, sample r, streptococci and a Gram-negative rod isolated. (SEE TEXT PAGE 60).

PLATE 6a. A "L" form? Organism grown on MacConkey agar.
Gram's method of staining.
Oil-immersion, X 1,000.

PLATE 6b. "L" form? Organisms grown on MacConkey agar. Culture 25. Gram Oil-immersion, X 2,250

PLATE 6c. As Plate 7b. Gram. Oil-immersion, X 2,250

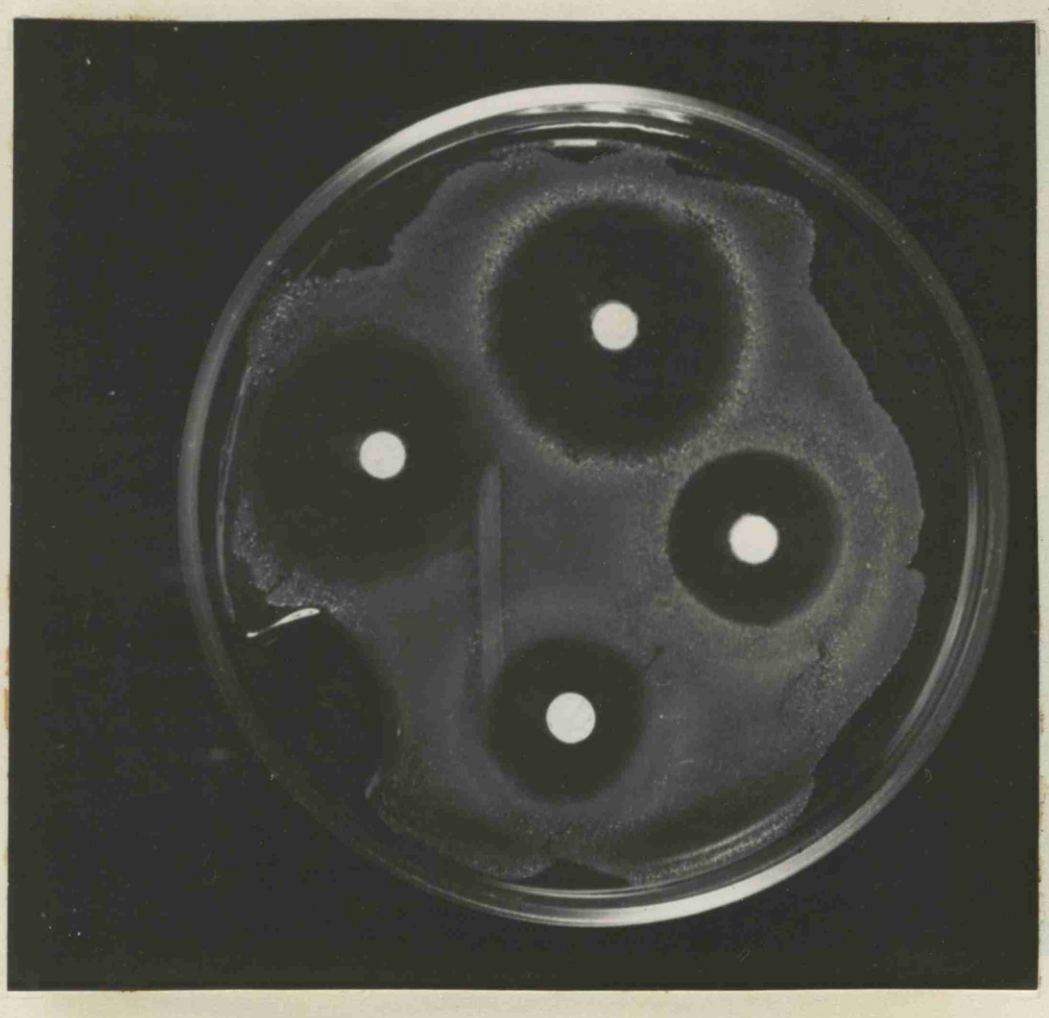

PLATE 7. Penicillin-sensitive Staph.aureus from milk.
Antibiotics tested:- penicillin, erythromycin,
chlortetracyline, streptomycin.

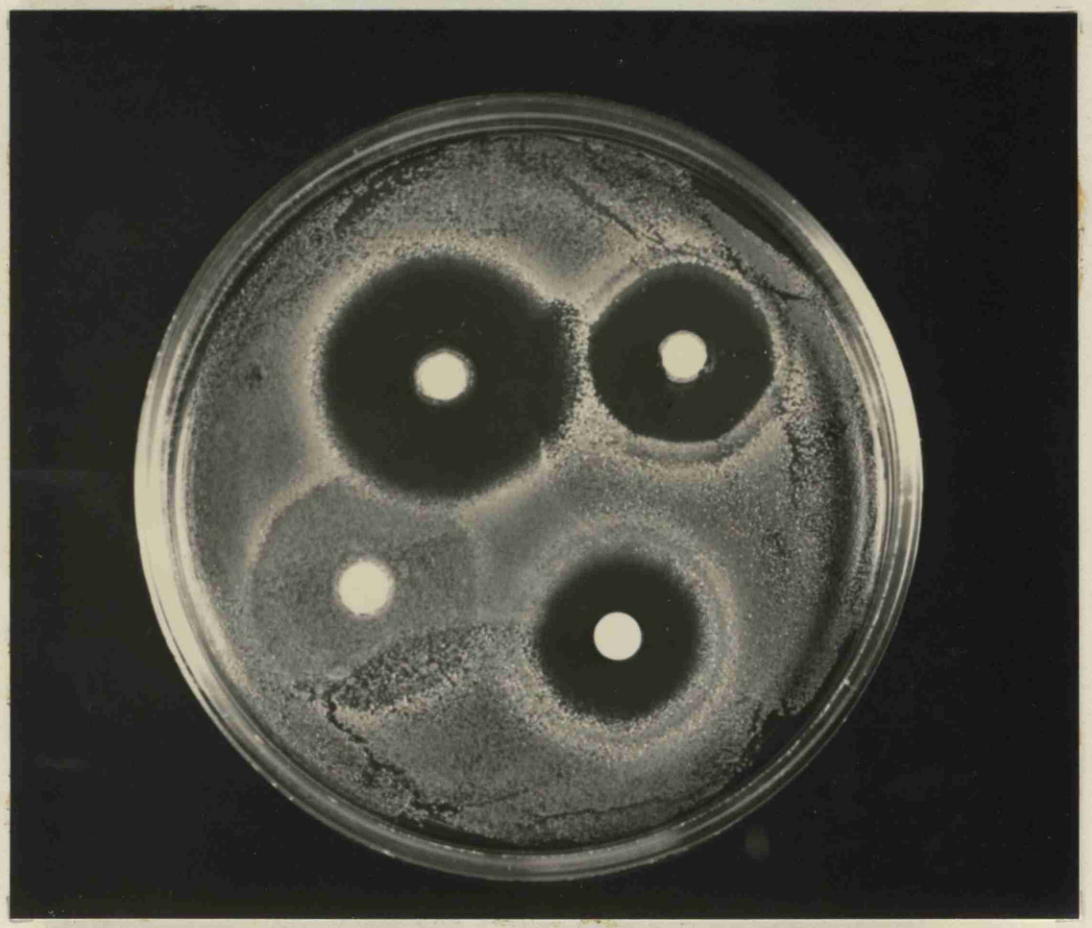

PLATE 8. Penicillin-resistant Staph.aureus from milk. Antibiotics tested:- penicillin, erythromycin, chlortetracycline, streptomycin.

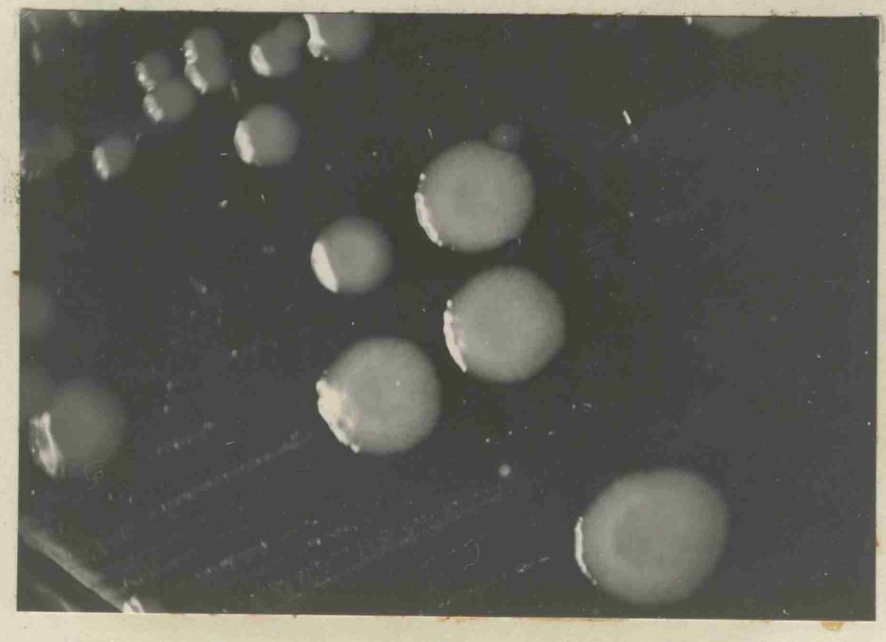

PLATE 9a. Colonies of E.coli on MacConkey agar, overnight culture at 37°C. Magnification X 4.

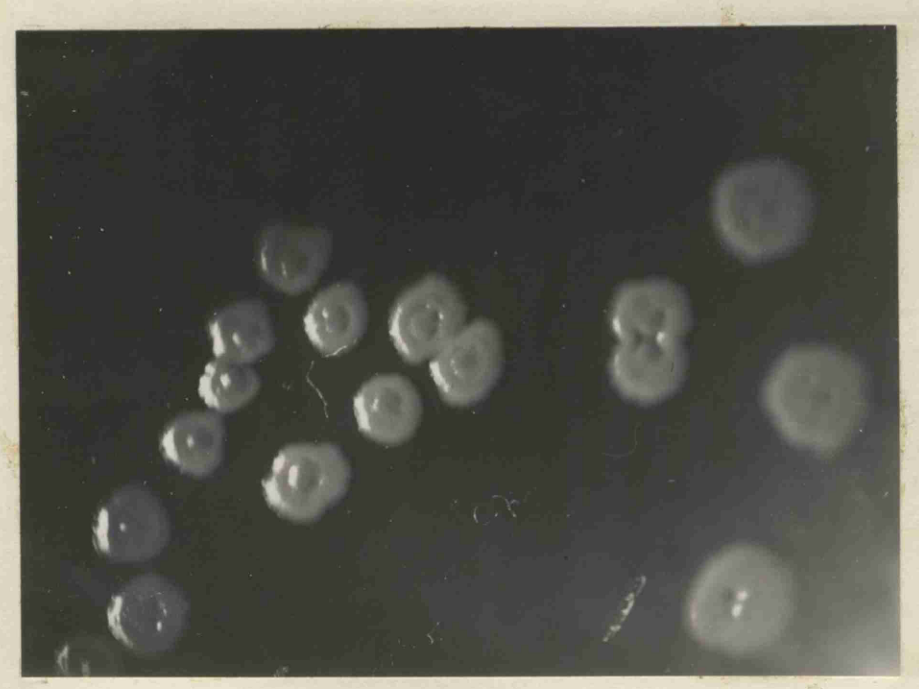

PLATE 9b. Colonies of culture 25 on MacConkey agar, overnight culture at 37°C. Magnification X 4.

PLATE 10a. Culture 25, grown on nutrient agar. Normal morphology.
Gram Oil-immersion, X 2,250

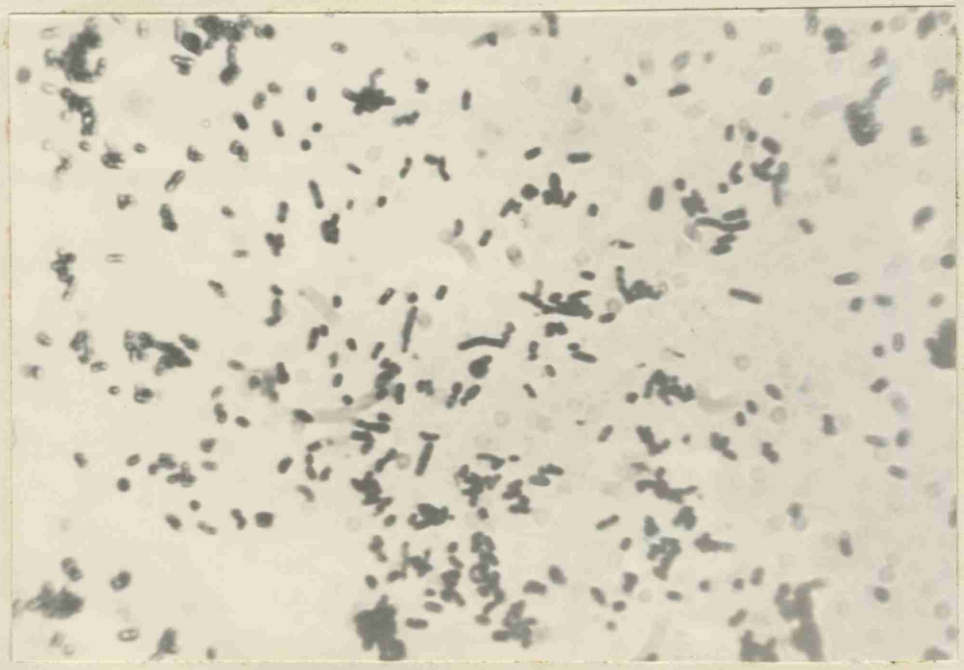

PLATE 10b. As Plate 10a. Gram Oil-immersion, X 2,250

PLATE 11. Colonies of Culture 25 on nutrient agar, overnight culture at 37°C. Magnification X 4