THE INCIDENCE OF BRUCELLAR INFECTION IN THE WEST OF SCOTLAND

WITH SPECIAL REFERENCE TO

THE MILK SUPPLY OF THE CITY OF GLASGOW.

A THESIS

submitted by

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C O N T E N T S

Summary./

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INTRODUCTION

Brucellosis is a contagio-infectious disease of man and of most species of domesticated animals. Occasionally, the condition is encountered in a wide variety of wild animals, such as the bison, buffalo, camel and several kinds of deer. The barnyard fowl and other birds also may become infected. Of the small laboratory animals, guinea-pigs are most susceptible to artificial infection whilst the brown rat (Rattus norvegicus) may become infected naturally as well as experimentally.

The disease is caused by a small, Gram-negative micro-organism which varies somewhat in size. Thus, it may occur as a coccus of 0.6 micron in diameter, as in the case of Brucella melitensis which was originally known as Micrococcus melitensis. (Fig.1). Again, it may assume the form of a short bacillus that measures 0.6 - 1.2 micron long by 0.5 - 0.7 micron broad. Brucella abortus and Brucella suis generally appear as short rods, 1 - 2 microns long, but coccoid forms also occur (Topley & Wilson, 1955), as indicated by Fig. 2. The genus is sub-divided into species, the designation of which derived from the country of origin, from the clinical character of the related disease or from the name of the animal from which the micro-organism was primarily

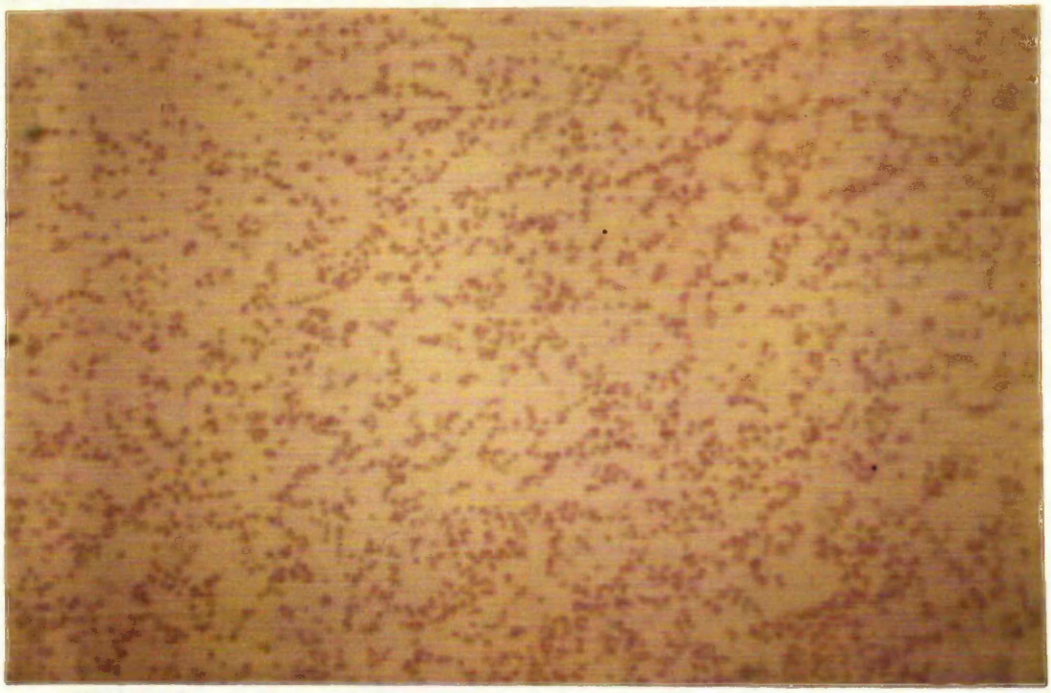

Fig. 1: Brucella melitensis (x 1330)
from growth on serum dextrose agar.

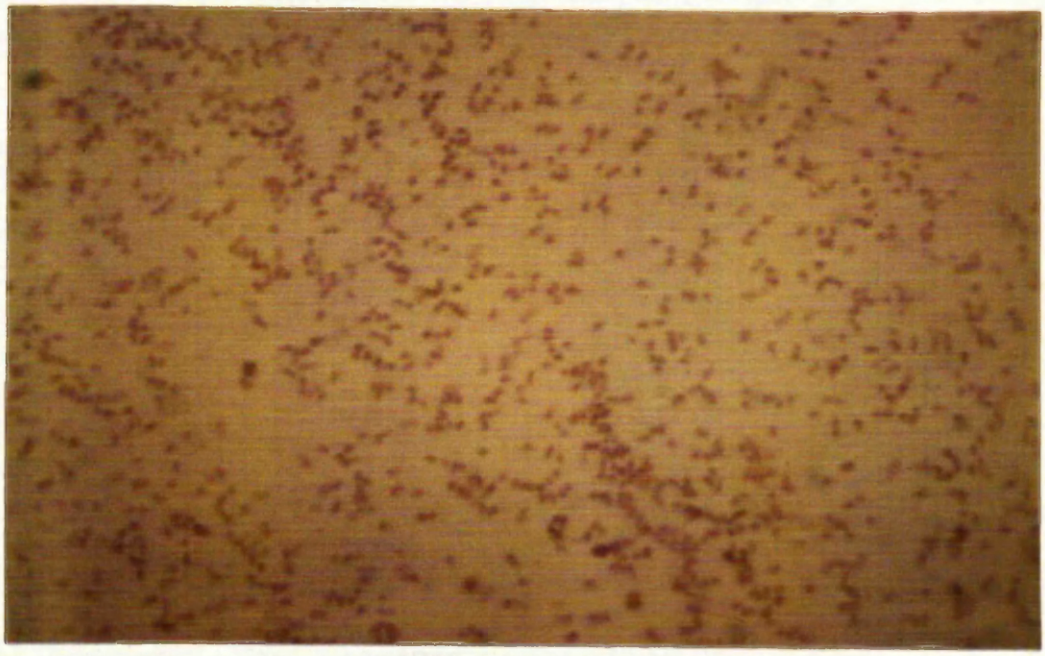

Fig. 2: Brucella abortus (x 1330)
from growth on serum dextrose agar.

primarily/

isolated. Br. melitensis, for instance, was first obtained from human beings on the island of Malta. Br. abortus was recovered from cases of abortion in cattle and Br. suis is the designation reserved for those strains which are peculiarly pathogenic to the pig.

More recently, Br. ovis is the name which has been proposed for a Gram-negative micro-organism that has been associated with a genital disease of sheep reported from New Zealand by Buddle & Boyes (1953) and from Australia by Simmon & Hall (1953). According to Blood & Henderson (1963), a similar condition is present in The United States of America and in South Africa as well as in Europe.

From the desert wood rat (Neotoma lepida, Thomas), Steonner & Lackmann (1957) isolated a new species of Brucella which has been designated Br. neotoma and which, according to Beal et al. (1959), is serologically related to Br. abortus and to Br. suis.

Humans and animals alike are susceptible to all three classical species although such susceptibility varies considerably. Br. melitensis appears to be most pathogenic to man in whom it causes a condition known as Malta, or undulant fever. That micro-organism, however, is most widely spread among goats and sheep in the Mediterranean littoral, in South Africa, in some parts of the U.S.A. and in Russia. In newly infected animals, it may sometimes provoke clinical illness with wide-spread abortion but usually is associated with chronic illness, accompanied by arthritis and unthriftiness. In goats, especially,

especially/

clinical signs may be lacking yet the micro-organism is excreted in enormous numbers in the milk and in the urine. Occasionally, in certain countries, Br. melitensis has been recovered from bovine milk.

Although Br. abortus is generally considered to be of low virulence for man, it is nevertheless very widely encountered throughout the world. It is the causal agent of "Contagious Abortion" of cattle and is the species mainly responsible for cases of brucellosis that occur in this country. As yet, neither Br. suis nor the so-called Br. ovis nor Br. neotoma have been described in Great Britain.

Brucellosis is of world-wide occurrence and its significance is two-fold, namely:

(a) It causes serious economic losses to the animal industry and

(b) It constitutes an important public health problem.

A. ECONOMIC IMPORT

Losses from brucellosis in cattle arise mainly from decreased milk-yield, loss of calves, effects on fertility and interference with the desired seasonal sequence of calving. According to British and American investigators, the decrease in milk-yield associated with early abortion may be up to 40 or 50 per cent, whilst cattle aborting at a late stage of pregnancy furnish about 20 per cent less milk (Minett & Martin, 1936). The quality of the milk secreted by Br. abortus-infected udders is also inferior to that of healthy udders, (Bryan & Meyer, 1936).

1936./

Gow & Hamilton (1935) studied over a period of four years 74 Brucella-infected cows as well as 222 animals which were free from such infection and gave the following figures:

	(In pos. reactors)	(In neg. reactors)
Services per conception	1.79	1.48
Calves per year	0.86	1.07
Fat in milk	251.6	281.6
Milk per cow	6,330 lb.	6,884 lb.

In the U.S.A. a reduction by half in the incidence of brucellosis of cattle resulted in a saving of 50 million dollars per annum, (W.H.O./F.A.O Report, 1951). That publication also contained evidence that the total cost of eradication of bovine brucellosis in Norway was less than the annual economic loss formerly caused by the disease.

In 1960, Mingle reported that a programme of eradication in the U.S.A. had reduced the estimated annual losses due to brucellosis from 100 million dollars in 1947 to less than 30 million dollars. McDiarmid (1960b) estimated that losses due to the disease were of the order of £16,250,000 for England and Wales.

Among animals, infection may be of sub-clinical type, particularly where calfhood vaccination with Strain 19 is practised. Thus, the old problem of "Contagious Abortion" in cattle may not any longer exist. Yet, a clinically healthy carrier animal, that yields infective milk or excretes the pathogen in the utero-vaginal discharge, constitutes a distinct risk for other animals as well as for man. Year by year, there is an increasin

increasing/

awareness of that problem, for which the only remedy is eradication of the disease.

B. THE PUBLIC HEALTH PROBLEM

Nigh on sixty years ago Horrocks (1905) pointed out that ingestion of infected goat's milk was an important mode of infection in man and, from an analysis of a series of 1,134 cases, Dalrymple-Champneys (1953) concluded that the most important source of human infection in Great Britain was raw cow's milk or products derived therefrom.

The clinical manifestations of human brucellosis vary considerably whereby the condition is often difficult to diagnose. A fatal case of human brucellosis in the North of Scotland was reported by Marr (1933) who stated that, two days before death, the serum of the patient agglutinated both Br. abortus and Br. melitensis to a dilution of 1 in 51,600. Out of 900 patients with undulant fever Dalrymple-Champneys (1960) was able to make a provisional diagnosis in 148 cases.

In this country the disease is not notifiable and there are few, if any, accurate statistics of its incidence although Dalrymple-Champneys (1950) estimated that about 1,300 human cases of brucellosis occurred annually in England and Wales. Those people became infected by consumption of unpasteurised bovine milk or of dairy products derived from infected animals or by contact with such animals or their tissues.

The magnitude of Brucella infection of man may be deduced from the words of Sir Weldon Dalrymple-Champneys who studied the problem for

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over 30 years and published in 1960 a book with the following inscription: "To all patients past and present with undiagnosed undulant fever this book is dedicated".

In 1958, the World Health and the Food and Agricultural Organisations recommended that the determination of the extent to which living Brucellae occurred in food products destined for human consumption was a matter that demanded immediate investigation throughout the world. In October, 1960, there was instituted a national survey of brucellosis in dairy herds which aims to determine the national incidence of herd infection, the relation of uterine infection to that of milk, the effects of the use of S.19 vaccine and, finally, the extent of the economic loss caused by brucellosis of cattle.

Such an investigation may be regarded as a first step towards eradication of the disease from British herds. The study undertaken by the writer was designed to determine the incidence of Brucella infection in milk supplied to the City of Glasgow and, to the best of his knowledge, there is not any other report of its kind. The work involved was carried out during the period, February 1961, to September 1963, and was divided into the following three parts:

Part I. The isolation of brucellar micro-organisms from samples of milk.

Part II. The typing of recovered brucellar strains together with a study of their viability and of their dissociation in artificial cultur

Part III. The demonstration of brucellar bacteriophage in laboratory cultur

PART I. THE ISOLATION OF BRUCELLAR MICRO-ORGANISMS FROM SAMPLES OF MILK.

1. Historical.

2. Materials:

   Group 1. Samples of certified milk.

   Group 2. Samples of milk taken from pasteurizing plants.

   Group 3. Samples of milk drawn for aborting animals.

   Group 4. Samples of milk obtained from individual animals of a certified herd and associated with a case of human brucellosis.

3. Methods:

   A. Serological tests:

      1. The milk-whey tube agglutination test.
      2. The milk ring (or Abortus-Bang ring) test.
      3. The milk capillary tube test,
      4. The milk plate agglutination test.

   B. Biological examination.

   C. Cultural examination.

   D. Microscopical examination.

4. Results.

5. Discussion.

6. Summary.

1. HISTORICAL

Ever since he began to domesticate the lower creatures and to breed them for the purpose of food production as well as for exploitable energy, man has been interested in animal reproduction. Gradually he has come to realise the difficulties and the practical problems which are

are/

so involved. Thus, farmers and stock-breeders are all too conscious that domestic animals may cast their young and give birth before the normal time, an event which was formerly attributed to accident or to environmental causes.

The belief that prevailed about abortion and other breeding difficulties passed from generation to generation of farmers until 1567 when, according to Hutyra and Marek (1922), Mascall called attention to the disease in England. Early in the nineteenth century, the contagious character of the disease was known to farmers who observed that often the condition spread from animal to animal in a herd. According to Nocard and Leclainche (1905), as far back as 1804, Flandrin stated that, so convinced were they of the contagious nature of abortion of cattle, that peasants carefully covered up a foetus and removed it from a byre through a window lest a cow later traverse the same route.

in 1806, the English agricultural press deposed that abortion of cattle might be contagious and advised that animals so affected should be isolated. In 1826, however, Hurtrel d'Arboval in France said "One is not content to believe it epizootic" and in England Youatt (1834) declared "There is no contagion". Hering (1858) wrote "There are periods when abortion in some herds occurs frequently and widespread, so that it can not be explained by chance and local causes". Robert Jennings (1864), in his discussion on "Cattle and their diseases", was of the opinion that abortion was due to some sympathetic influence, i.e. if a pregnant cow

cow/

were to see another animal aborting, she herself would abort within a few days or some weeks. Hence he recommended that the disease be controlled by isolation of aborting animals from the others.

According to Mutyrâ & Marek (1922), during the last century the disease was considered by Lawrence & Skellet to prevail in contagious form in several mountainous districts of England. Such a foothold did the condition gain in dairies of certain localities that 50 to 60 per cent of the cows slunk their calves. Zundel (1871) and St. Cyr (1875) maintained that an infective agent was the causative factor, in spite of the opposite view held by Mutrel d'Arboval, Hering, Stockfleth and others. Even as late as 1878, Stockfleth refuted the infectious nature of the disease and said, "Such a theory is surely very convenient in that it lets us ignore other real causes which it can be very difficult to establish and abolish". On the other hand, previous observations by several French veterinary surgeons tended to sustain the infectious character of the disease (Traite d'Obstetrique Veterinaire 1875).

However Franck (1876) and, later, Lehnert (1878) and Brauer (1880), reproduced the disease experimentally by the introduction of utero-vaginal discharge and of portions of foetal membranes per vaginam into healthy cows.

Nocard (1886) was the first to undertake a close study of the disease and tried to isolate the causative agent. From the exudate

exudate/

found between the uterine mucous membrane and the foetal membranes, he recovered a micrococcus and a short bacillus, but failed to reproduce the disease by inoculation of pure cultures of either of those organisms into healthy pregnant animals.

For artificial culture, Nocard made use of peptone-gelatine or of peptone-water but, as we now know, neither of those media support the growth of brucellae. Despite his failure to reveal the causative organism, Nocard made some valuable clinical observations which indicated that bovine abortion was not infrequently of infectious origin.

In a report to the Highland and Agricultural Society of Scotland, Woodhead, Aitken, McFadyean and Campbell (1889) reported that abortion could be reproduced in cows and ewes by inserting into the vagina cotton-wool plugs that had been contaminated with the utero-vaginal discharge of aborting cows. They also claimed to have brought about the abortion of a living foetus as a result of subcutaneous injection of the utero-vaginal discharge taken from another aborting bovine. Sand (1894) emphasized the infectious character of the disease and gave various clinical data.

Since 1897, the history of the disease falls into two important stages, which need here to be separately discussed.

discussed./

A. THE ISOLATION OF BACTERIUM ABORTUS AND TWO OTHER MEMBERS OF THE GENUS BRUCELLA FROM PATHOLOGICAL SPECIMENS.

In 1897 Bang & Stribalt recovered the causative micro-organism culturally from the yellowish, odourless uterine exudate of aborting cattle. They succeeded in reproducing the disease artificially by introducing a pure culture of the bacillus into the vagina of two pregnant cows. From both of the experimental animals they later recovered the same micro-organism and so fulfilled Koch's postulates. Bang's work was corroborated by Preisz (1903) in Hungary, Nowak (1908) in France, McFadyean & Stockman (1909) in Great Britain, Zwick (1910) and MacNeal & Kerr (1910) in America, Holth (1911) in Germany and other investigators elsewhere in the world.

Ten years before Bang and Stribalt reported their findings, Micrococcus melitensis had been recognized as the cause of Malta fever of man. It was isolated by Bruce (1887) from the spleen of a soldier who had died of Malta fever.

A close morphological, biochemical and serological relationship between Micrococcus melitensis and Bang's Bacillus was demonstrated by Evans (1918). Her observations were soon confirmed by Meyer and Shaw (1920) and by Feusier and Meyer (1920) and, in honour of Sir David Bruce the generic name "Brucella" was proposed by the two former workers. The term, brucellosis, was suggested in analogy with

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Tuberculosis to apply to all types of Brucella infection of men and animals (Topley & Wilson 1955). Evans (1925) also suggested that the term Brucella should properly be abbreviated to "Br." since "B" was the contraction already used for the genus Bacillus. In that paper Evans stated that she had induced experimental abortion in the cow by inoculation of a culture of Br. melitensis isolated from a human case of Malta fever and that she had recovered the same strain from the foetus and the colostrum.

In 1914 Traum recovered from aborting sows another species of micro-organism which, morphologically and serologically, proved to be closely related to Br. abortus and was later named Brucella suis. Its natural habitat is the pig and man may become infected mainly by contact with either diseased carcases or infected animals (Topley & Wilson 1955). As a result of contact with infected pigs, cattle and other species of animals may acquire the disease, and Br. suis may occasionally be transmitted to man from creatures other than the pig.

The primary growth of Br. abortus and its behaviour towards atmospheric oxygen presented certain difficulties during artificial cultivation. Bang (1897) first observed that the organism grew in a dense zone situated about 0.5 cm. below the surface of a semisolid medium and to a depth of 1.0 - 1.5 cm. To overcome the difficulties that attend primary isolation, Preisz and Nowak altered the method of

of/

cultivation. Preisz (1903) seeded utero-vaginal discharge on three ordinary agar slants and utilized pure oxygen to replace the atmosphere of all the culture-tubes, which latter were at once made air-tight with sealing-wax. After incubation at 37°C. for three days, one culture was found to contain a few minute and scarcely visible colonies which consisted of small rod-shaped bacteria that morphologically resembled Bang's bacillus. Nowak (1908) demonstrated that visible growth was obtainable from infective material incubated in closed containers that included cultures of Bacillus subtilis. He thought that the latter bacterium used up so much of the free oxygen in the container that there was created the degree of reduced oxygen-tension essential to the growth of Br. abortus. Those observations were interpreted to mean that the micro-organism was microaerophilic and required for its growth a lower oxygen-pressure than is normally present in the atmosphere. However, methods of cultivation remained disappointing until 1919 when Huddleson, Hasley and Torrey began a study of the physiology of Br. abortus (Huddleson et al., 1927), which eventually revealed that the rapid growth and brisk respiration of B. subtilis gave rise to an increased tension of carbon dioxide sufficient to promote the development of cultures of Br. abortus that had been placed in the same container. The actual volume of carbon dioxide in the receptacle was found to average 10 per cent, an amount

amount/

which closely approximated that of the gas present in the fluids of the udder and of the pregnant uterus. When a like quantity of carbon dioxide was introduced artificially into jars containing culture-media seeded with infective foetal gastric contents, a heavy growth of Br. abortus was obtained after incubation at 37°C. for 48 to 72 hours. Further work by McAlpine & Slanetz (1928) confirmed the importance of an atmosphere charged with carbon dioxide.

At first, largely because he had proved it, Bang thought that the only way to infect a cow was by means of the introduction of Br. abortus into the vagina of a pregnant animal. He suggested that copulation afforded the most favourable opportunity for transmission and that bulls were the only carriers capable of introducing the micro-organism directly into the uterus. Later, in 1906, he carried out further experiments, during which infection was established by inoculation into the blood as well as administration via the alimentary tract and other routes.

B. THE DEMONSTRATION OF BRUCELLA MICRO-ORGANISMS IN GOAT'S AND IN COW'S MILK.

Following the discovery by Bang (1897), many investigators such as Grinstadt (1910), Wall (1911), Holth (1909), Brull (1911), Larson (1911) and others, concerned themselves particularly with methods applicable to diagnosis of the disease in infected cows.

cows./

Schroeder (1894) had recorded the presence of unidentified lesions in guinea-pigs that had been inoculated with cow's milk suspected of containing tubercle bacilli, at which time the existence of Br. abortus was unknown. Zeller (1923) also reported that, in 1906-1909, in the course of similar investigations he had frequently encountered lesions of undetermined etiology which he had classified as "pseudo-tuberculous".

Horrocks & Zammit (1905) showed that four of their experimental goats harboured Micrococcus melitensis in the milk and that two of them excreted the bacterium in the urine as well. Horrocks and Kennedy (1906), two members of a commission of Mediterranean Fever, also cultivated Micrococcus melitensis from the milk of 10 per cent of the goats maintained in various parts of Malta. On the basis of serological tests, they finally concluded that 40 per cent of the goats on that island were naturally infected by the pathogen and that man acquired the disease through the medium of raw milk. The presence of specific agglutinins in the serum of animals experimentally infected by Micrococcus melitensis as well as in the blood of human patients had already been demonstrated by Wright and Semple (1897), a finding that was confirmed subsequently by Birt & Lamb (1899) and by Zammit (1900).

Prior to 1911, aborted foetuses and utero-vaginal discharges containing Brucellae were considered to be the main sources of infection

infection/

for cattle but the work of Schroeder & Cotton (1911) and of Mohler & Traum (1911) proved that Br. abortus may occur also in the milk of infected cattle. By inoculating cow's milk from 31 dairies into guinea-pigs, Schroeder & Cotton (1911) found that eight out of 77 milk samples derived from 6 dairies contained Br. abortus. Thus, raw milk infected by Br. abortus came to rank as another important source of infection. Speedily those observations were confirmed by Smith & Fabyan (1912) and soon afterwards by Zwick & Krage (1913) and by Fleischner and Meyer (1917).

Although Schroeder & Cotton (1911) at first described as "Gram-positive" the minute bacillus which they cultivated from guinea-pig lesions, later work proved that the micro-organism was actually Bang's Bacillus. Mohler & Traum (1911), who worked independently, reported the micro-organism to be Gram-negative.

The finding of Bang's Bacillus in samples of bovine milk focussed attention on the following problems:-

(a) the possibility of the spread of infectious abortion among cattle through the medium of milk,

(b) the effect of the infection on the udder and the yield of milk, and

(c) the likelihood of the transmission of infection to man.

Cotton (1913-1914) reported that the elimination of Bang's bacillus in bovine milk was persistent.

persistent./

Evans (1915) published a short note to indicate that she had found Bang's Bacillus in approximately 30 per cent of bovine milk samples taken from two certified herds near Chicago. In 1916 and in 1918, the same author recorded the cultivation of the bacillus from samples of milk procured,with due precautions from individual cows. She used plain infusion agar to which 10 per cent of sterile bovine blood serum had been added. The recovered pathogen was subsequently identified by means of agglutination with positive antisera. Fleischner and Meyer (1917) reported that Bang's bacillus was present in certified milk produced in the vicinity of San Francisco Bay.

Until 1911, the diagnosis of Bang's disease was based on clinical signs corroborated by demonstration of the presence of agglutinins in the blood of infected cows as well as by microscopical or cultural disclosure of the causative organism in the after-birth, the utero-vaginal discharge or the gastro-intestinal contents of the foetus. The main pathological features of the disease are endometritis and placentitis that result in abortion.

More recently, the biological method of diagnosis has been widely used since it not only affords a more delicate means of detecting Brucella in infected milk but is also particularly useful when the number of eliminated micro-organisms is so small that they fail to be revealed by cultural methods. Again the biological method

method/

is of singular value in the case of milk samples which have become grossly contaminated by other micro-organisms.

The close antigenic relationship between Br. melitensis and Br. abortus, originally demonstrated by Evans (1918), suggested the likelihood of the transmission of infection to man. Although Weil and Menard (1912) had reported the case of a Parisian who, during holiday in a country district where Mediterranean fever was unknown, had daily consumed a litre of cow's-milk and subsequently contracted brucellosis, Bevan (1921) was the first to demonstrate an agglutinating titre of 1/200 for Br. abortus in the blood serum of a patient with brucellosis in Southern Rhodesia, where contagious abortion of cattle was common. In 1924, Keefer announced that, two years previously, he had isolated an organism belonging to the Br. abortus group from the blood of a patient in Johns Hopkins Hospital. Bevan's observation was duly confirmed by Orpen (1924) in Rhodesia, by Dalrymple-Champneys (1929) in England and by later reports emanating from nine European countries as well as from Palestine and from Canada, (Dalrymple-Champneys 1950). Those findings led investigators in different parts of the world to use biological and serological tests on bovine milk as means for the diagnosis of brucellosis of cattle.

In Australia, Seddon (1919) recorded the isolation of Br. abortus via the guinea-pig from the milk of 37 out of 52 cows (71 per cent), the sera of which latter contained specific antibodies.

antibodies./

Huddleson (1920) described a simple cultural method for the isolation of Brucella from the cream of milk that had been kept in a cold room for 24 hours but that procedure was of value only in the case of hygienically collected samples. He stated that contaminated milk might be successfully examined for brucellosis by means of the inoculation of guinea-pigs. Carpenter (1926) recovered Br. abortus from the milk of three previously vaccinated cows which had given negative reactions at a serum dilution of 1/60.

In Manchester, Wilson and Nutt (1926) employed guinea-pigs in the examination of 488 samples of bovine milk and found thereby that 5.7 per cent of individual samples and 8.8 per cent of mixed samples contained Br. abortus. By the same means, Carpenter and Baker (1927) investigated the milk from fifty herds that supplied the town of Ithaca, New York, and discovered that nine were afflicted by Br. abortus. King (1928) recorded the case of a cow, the milk of which contained Br. abortus yet its serum proved negative to the tube agglutination test. Gilman (1930) examined milk from all the quarters of 34 cattle for the presence of agglutinins and subsequently inoculated milk sediment and cream into guinea-pigs. Br. abortus was recovered from 53.7 per cent of the quarter samples that contained agglutinins to a dilution of 1/80, or more, but not from those with a titre of under 1/80. He also failed to demonstrate the presence of Brucella organisms in the milk of any animal with a blood-titre lower

lower/

than 1/320. Wall (1930) found Br. abortus to be present in the milk of cows which had previously aborted. He inoculated the cream and the milk sediment separately into guinea-pigs and so determined that cream contained more Brucella organisms than did the sediment after centrifugation. Hasley (1930) examined by cultural means 230 samples of certified milk obtained from five dairies in the city of Detroit and contained in bottles ready for delivery. From ten of them Br. abortus was isolated in numbers that ranged from 2 to 8 micro-organisms per millilitre of milk. Bang & Bendixen (1931) studied the milk from 42 quarters of 11 cows, taken just after calving, and found Br. abortus to be present in 31 of them, most of which had been drawn from posterior quarters. The authors emphasized that the physical character of the milk was not altered even in those cases in which as many as 30,000 micro-organisms were present per millilitre of milk. Gilman (1931) examined the milk from all four quarters of 113 cows for the presence of both agglutinins and Br. abortus itself. By means of inoculation into guinea-pigs of the sediment after centrifugation the micro-organism was recovered from 62.9 per cent of the cows that had a positive blood titre of 1/80 and from 78 per cent of those with a milk-whey titre of 1/80. Again by guinea-pig inoculation, Wood & Illing (1931) tested mixed samples of milk derived from 379 dairy herds in the county of Somerset and demonstrated that Br. abortus was present in four of them. 10 per cent of 2079 mixed quarter samples of milk

milk/

scrutinized by Schmidt (1932) proved to contain Br. abortus. In Edinburgh, Beattie (1932) examined three groups of samples which consisted of pasteurized, retail and raw milk, respectively. None of the 66 pasteurized samples inoculated into guinea-pigs yielded Brucella but 17 out of 86 raw samples (19.8 per cent) and 29 out of 83 retail samples (34.9 per cent) were so positive and the micro-organism was recovered from the spleen of the experimentally infected cavies. In Aberdeen, Smith (1932b) tested samples of raw and of pasteurized milk. Of 279 raw samples 79 (28.3 per cent) contained Br. abortus whereas none was recovered from 187 samples of pasteurized milk collected from plants in which the "Holding" method was employed. On the other hand, Br. abortus was recovered from 36 (18.7 per cent) out of 192 samples subjected to the "Flash-point" treatment, during which the milk is held momentarily at a temperature of 145°F. Morgan (1932) reported that of 76 routine samples of mixed milk examined by the method of guinea-pig inoculation 28 (37 per cent) were positive. In Burnley, Priestley (1932) inoculated 760 guinea-pigs with samples of milk primarily for the diagnosis of tuberculosis and found Brucella agglutinins to occur in the blood of about 20 per cent of the animals, from the lymph nodes, spleen and heart's blood of which Br. abortus was subsequently isolated. Henry, Traum & Harring (1932) used cultural and biological methods for the isolation of Br. abortus from bovine milk and concluded that guinea-pig inoculation was

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the more accurate since, per se, the cultural method failed to detect Brucella in from 25 to 30 per cent of the cases that proved positive by the biological method. In New Zealand, Hopkirk and Gill (1933) inoculated 642 guinea-pigs with composite samples of milk from herds supplying various towns and cities. Br. abortus agglutinins were later detected in the sera of 231 of them (36 per cent) whereas, by means of culture on gentian-violet serum-agar, the pathogen was recovered from only 13 (2 per cent) of the samples. Wohlfeil & Schulz (1933) surveyed 489 individual milk samples obtained from 17 different herds by use of a selective medium containing malachite green and gentian violet. Br. abortus was found in only 19 samples when cultures were made from milk that had not been previously centrifuged but, when the inoculum consisted of the sediment after centrifugation, 29 samples yielded a growth of micro-organism. The authors did not recommend the cultural method for the isolation of Brucella micro-organisms from group milk samples. Gaiger & Davies (1933) tested 200 bovine milk samples by guinea-pig inoculation and found that 52 (26 per cent) contained Br. abortus. Of 489 samples of milk examined culturally by Stockmayer (1933) 29 yielded the pathogen but 22 per cent of the plates were contaminated by other micro-organisms. Plate (1933) demonstrated, by direct cultivation and by guinea-pig inoculation, that out of 81 milking cows, 56 (69.1 per cent) excreted Br. abortus. Caldwell et al. (1934) found by biological means that the pathogen was

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eliminated in the milk of 25 out of a group of 163 cows. By the same procedure, Smith (1934) proved that of 287 milk samples from individual cows belonging to a single herd 33 (11.5 per cent) contained Br. abortus and that out of another 203 random samples obtained from individual cows Br. abortus was isolated from 23 (11.3 per cent). Thompson (1934) examined the milk of ten apparently normal cows by both guinea-pig inoculation and cultural methods and found that six contained Br. abortus. Plate (1934) examined biologically 413 samples of mixed milk from individual cows and established that of 28 samples, which gave a weakly positive reaction to the rapid slide agglutination test carried out with whey, 12.1 per cent contained Brucella micro-organism whilst 49 serologically positive samples and 53 with a like pronounced positive reaction yielded Brucellae to a percentage of 61.2 and 88.7, respectively. Of a total of 842 group milk samples examined by the same author Br. abortus was found in 2.3 per cent. Out of 709 samples, which by the rapid slide whey agglutination test proved to be free from specific agglutinins together with another 35 milk samples that gave a non-specific reaction, Brucellae were detected in 2.7 per cent. On the other hand, of 40 weakly positive and of 52 positive samples Brucellae were recovered in 7.5 and 32.7 per cent, respectively, whilst of 6 samples, strongly positive by slide agglutination, the pathogen occurred in 3 (50 per cent). Karsten & Bichoff (1934), in a biological study of 187 milk samples

samples/

obtained from naturally infected cows, detected Brucella in 98.4 per cent whilst by cultural means they isolated the micro-organism from 41.3 per cent. Pullinger (1935) examined 31 samples of raw cream by inoculation of guinea-pigs and found that 11 contained Br. abortus. According to Graham & Torrey (1936), Brucella micro-organisms were isolated from about half of 62 samples of raw milk collected from 28 dairies in the North American State of Illinois, whereas 31 samples of pasteurized milk proved to be free from the pathogen. Guinea-pig inoculation was used also by Doyle & Bekett (1936) in application to samples of milk drawn from 309 cows which belonged to 17 infected herds and which had proved negative to the blood agglutination test for bovine Brucellosis. The authors considered blood-titres of less than 1 in 25 to be negative and stated in conclusion that "Br. abortus was isolated from the milk of two cows which had negative blood titres in dilutions of from 1/25 to 1/200". Such a declaration is inexplicable, especially as in another paragraph Br. abortus was reported to have been isolated from the milk of a cow with a positive serum reaction at a dilution of 1/25. Fitch & Bishop (1937) examined raw milk from 67 dairies and isolated Br. abortus via guinea-pigs from 17 (25.4 per cent). In an attempt to establish the incidence of Br. abortus in the market milk of Zagreb and district, Forsek (1939) subjected 318 samples to the whey tube agglutination test, to cultural investigation and to guinea-pig inoculation. A

A/

positive agglutination reaction was observed in the course of nine samples, from six of which Br. abortus was recovered via guinea-pigs but only two of which were positive by cultural means. Menton (1940a) subjected 1,282 milk samples to both whey agglutination and biological tests. 34.5 per cent were revealed to contain Brucella agglutinins and 29.1 per cent of that group yielded Br. abortus. In addition, the pathogen was found to be present in 9.1 per cent of the samples that were devoid of agglutinins. During another investigation, Menton (1940b) recovered by the biological method an aerobic strain of Brucella from a sample of mixed bovine milk forthcoming from an accredited herd. Smitmans & Eschbaum (1941) inoculated guinea-pigs with mixtures of milk sediment and gravity cream and found that 222 out of 603 samples contained Br. abortus. Lewin et al. (1948) reported that of 212 raw milk samples 70 per cent contained Brucella agglutinins and that of 92 samples subjected to biological examination three yielded Brucellae. In a survey covering the Canton of Zurich, Hess & Sackmann (1953) found that 13 per cent of the herds and 6 per cent of all the cattle were affected by brucellosis and that the pathogen was present in the milk of about half of the infected herds. The authors investigated bulk milk samples by means of the milk ring test and quarter milk samples by use of the agglutination test and by cultivation. Huddleson & White (1954) isolated Br. abortus, type 2 Wilson, for first time from the milk of 5 naturally infected dairy

dairy/

cows in one herd in Michigan. In Edinburgh, Ferguson & Robertson (1954) examined samples of bulk milk drawn from 370 herds and found that 10.5 per cent contained Br. abortus. Sackman (1954) isolated 108 virulent strains of Br. abortus from the milk of cows previously vaccinated with Strain 19. Cultural and biological methods applied by Maestron (1955) to the milk of 1696 cows in the province of Milan revealed that 6.2 per cent of the animals possessed agglutinin that was detectable in the whey. 73.5 per cent of the latter creatures eliminated Brucella in the milk. Meir (1955) obtained Br. abortus from 85.3 per cent of bulk milk samples from infected herds by guinea-pig inoculation and from 80.7 per cent by means of culture. As a result of a two years' survey, Marr & Williams (1958) reported that samples originating from 25 per cent of the dairy herds in the Northern Counties of Scotland were positive to the Br. abortus milk ring test but that the pathogen was recoverable from only 25 per cent of such specimens. Borger (1958) isolated Br. abortus from 12 out of 25 herds in Schleswig-Holstein. In the Report on the Animal Health Services in Great Britain for 1959, out of 29 herds investigated 20 were stated to be infected by Br. abortus, which micro-organism was reported to have been recovered via guinea-pigs from 115 out of 118 samples and by culture from 103 out of 121 samples. Stableforth & Galloway (1959) in reference to the work of Cotton and his co-workers on Br. abortus, Strain 19, declare that

that/

the micro-organism was originally isolated from milk but the writer of this thesis was unable to find confirmation of that statement in the papers published by Cotton (1932) and by Cotton, Buck & Smith (1933 a and b, and 1934 a and b). Robertson (1961) recovered, by guinea-pig inoculation, 191 strains of Brucella from bovine milk derived from 158 herds. 171 strains were classified as Br. abortus, 16 were given as Br. melitensis and the remaining 4 were declared to be related serologically to Br. melitensis but biochemically to Br. abortus. According to Anon. (1961), of 11,450 herd samples of milk examined in 35 Public Health Laboratories located throughout England and Wales 544 (4.8 per cent) were found to be infected by Brucella. Of 390 cultures recovered from the same sources 93.6 per cent were typed as Br. abortus, and the remainder as Br. melitensis. According to Brodigan et al.(1961), during the period, 1957, 1959, Br. abortus was isolated from the milk produced by 25 dairy herds, comprising about 40^{0} head of stock. In another investigation, made by the same authors during 1958 and 1959, the milk ring test, the milk whey tube agglutination test and guinea pig inoculation were all used to explore samples of churn milk drawn from 520 registered dairy herds and reported that 33 (6.3 per cent) of the samples were infected. Brodigan and his co-workers also reported that of individual samples taken from 338 cows in 20 herds 33 (9.76 per cent) proved positive by guinea-pig inoculation. Cultural and biological

biological/

examinations made over a period of 16 months by Parry (1963) on milk produced by a herd of 35 tuberculin-tested Jersey cows in Bucklow revealed that 13 (37.1 per cent) of the animals excreted Br. abortus in the milk.

2. MATERIALS.

Four groups of milk samples were obtained from different sources, as described below.

GROUP 1: SAMPLES OF CERTIFIED MILK. "Certified Milk" is the designation sanctioned by the Milk (Special Designations) Scotland) Order, 1951, for application to the mammary secretion obtainable from milch cows which have been officially ascertained to be free from tuberculosis. Such milk must not be treated by heat in any manner and, on the premises where it was produced, it must be cooled to a temperature not exceeding 50°F. (10°C.) immediately after it has been procured and forthwith distributed into "retail containers" which may not exceed one quart in capacity. Those receptacles, or their seals, must bear the words "Certified Milk" and vessel itself must prominently display the name of the premises on which the milk was produced. Qualitatively, any sample of certified milk taken after it has been cooled but before it has been delivered to the consumer shall not contain more than 30,000 bacteria per millilitre or any coliform bacteria in one-tenth of a millilitre. A producer of certified milk is licensed to

to/

sell that product by wholesale at, or from, the premises on which the milk was produced.

The samples were obtained either from distributors in various parts of the City or directly from producers. At various times, one or two bottles of milk, sealed with caps marked "Certified milk", were taken at the time of milking from each of 114 farms and brought forthwith to the laboratory. Samples acquired from distributors were collected soon after delivery.

The contents of each bottle were separately tested by serological means and, if similar results were forthcoming, milk samples from the same source were mixed together and used for the isolation of Brucella micro-organisms. In the case of milk taken from farms just before bottling, care was exercised whereby an approximately equal amount of sample was procured from each can that was produced on the premises. In that way, it could be reasonably assumed that the sample was a mixture of milk furnished by all the animals on the farm. In some instances, the time of milking was so late in the afternoon that the samples of milk were collected the following morning.

As a consequence of those methods of collection, it is claimed that the milk samples in this investigation represent those likely to have been bought by consumers, whether from farms or from dairies. The procedures were repeated up to three times in the case

case/

of premises whence previous samples had been found to give positive, or indeterminate, serological results and to have been biologically negative. The total number of milk samples in this group was 128 originating from 114 farms as already mentioned.

GROUP 2: SAMPLES OF MILK TAKEN FROM PASTEURISING PLANTS.

Since the samples were taken from two different types of pasteurising plants, two different methods of sampling had to be adopted, namely:

(a) For batch plants, in which the milk is heated to 61°C. or 65°C. for not less than 30 minutes and then cooled in individual batches in tanks. One milk sample was taken from each tank before pasteurisation and another after treatment by heat.

(b) For plants dependent on the "High Temperature Short Time" system, in which the raw milk runs continuously from the storage tank into a small balance tank whence, at a controlled rate of flow, a constant stream of milk is pumped into the pasteurising machine. There the milk is filtered and heated to 71-72°C. for not less than 15 seconds when it is immediately cooled to a temperature of, usually, 10°C. ere it is withdrawn through the bottling plant. The length of time taken by the milk to pass from the small balance tank to the outlet of the bottling plant is about 50 seconds. A sample of raw milk was taken before treatment by heat either from the small balance tank, which was

was/

easily opened, or from the tube which carried the raw milk to the pasteurising machine. Some fifty seconds later, a second sample was drawn from the tube carrying the pasteurised milk to the bottling plant. Those procedures were designed to ensure that a batch of milk was adequately sampled in both the raw and the pasteurised state. To cover most of the milk delivered to the pasteurising plant on a particular day, samples were extracted at hourly intervals and removed to the laboratory without delay. Samples were collected from pasteurising plants once or twice per week, depending on the amount of milk under treatment. On a number of occasions, the samples had to be stored overnight in a refrigerator at 4°C. ere they were tested on the following morning. Altogether, 128 samples of milk (78 raw and 50 pasteurised) were secured from 27 different plants. As a rule, the samples were collected into ordinary milk bottles which were supplied by the pasteurising firm concerned and had been sterilised by exposure to steam. Occasionally, bottles specially sterilised by dry heat were used to contain samples. All the work of collection was done by the writer himself.

GROUP 3: SAMPLES OF MILK DRAWN FROM ABORTING ANIMALS.

All were provided by practising veterinary surgeons and were taken from animals with a history of recent abortion, of which brucellosis was the suspected cause. The group consisted of 73 samples of milk drawn from 56 animals located on various farms in the West of Scotland, with

with/

the exception of three specimens that came from the North of Scotland. Quarter samples from six of those animals were separately examined by serological and by cultural means but, for the biological test, the samples from each animal were pooled.

GROUP 4: SAMPLES OF MILK OBTAINED FROM INDIVIDUAL ANIMALS OF A CERTIFIED HERD AND ASSOCIATED WITH A CASE OF HUMAN BRUCELLOSIS.

Those samples were drawn into sterilised bottles provided by the Pathology department of the University of Glasgow Veterinary School. In all, 51 milk samples were procured from 51 lactating cows in the herd.

The man involved was a medical physician who had contracted the disease through the consumption of milk forthcoming from the herd. By courtesy of the Medical Officer of Health, the further facts of the case may be summarised as follows. After illness at home lasting for two weeks and characterised by headache, feverishness and slight soreness of the throat, the patient was removed to hospital suspected of either entric or abortus infection. On admission, there was pyrexia which responded rapidly and completely to administration of achromycin. The Widal reaction for enteric fever was negative. By contrast, the titre of agglutinins for Br.abortus was 1 in 2000 and rose to 1 in 4000 eight days afterwards but, seven days later still, had declined to 1 in 1000. Altogether, the patient was in hospital for eleven days, during which period all attempts to recover Br. abortus were unsuccessful.

unsuccessful.//

3. METHODS.

Altogether 380 samples of bovine milk were collected for investigation and were given the following distinguishing numbers:

1 - 114, in the case of certified milk,

1R - 78R, do. bulk raw milk,

1P - 50P, do. pasteurised milk,

1A - 56A, do. milk from aborting animals and

1B - 51B, do. samples derived from a certified herd.

Repeat samples retained their original numbers. All samples were assayed by the following methods of survey:

A. Serological examination; C. Cultural examination and

B. Biological examination; D. Microscopical examination.

A. SEROLOGICAL EXAMINATION:

The selected methods comprised:

(1) The milk ring (or Abortus-Bang Ring) test;

(2) The milk plate agglutination test;

(3) The milk capillary tube test and

(4) The milk-whey tube agglutination test.

1. THE MILK RING OR (ABORTUS-BANG RING) TEST.

Originally described by Fleischhauer (1937) and Herman (1937) in Germany, the Milk Ring Test was introduced for the diagnosis of brucellosis in infected lactating cows and depends upon interaction

interaction/

between stained Br. abortus antigens and homologous antibodies present in the milk. Agglutination ensues and is possibly followed by adsorption of the clumped micro-organisms to the protein-membrane of the fat-globules of the milk. When the latter ascend to form a layer of gravity cream, a blue ring is produced at the surface of the fluid.

Since its inception, the milk ring test has come to be extensively used in different parts of the world, particularly Sweden, Denmark and The United States of America. Both the procedure of the test and the mode of preparation of the antigen have been improved by the Swedish workers, Norell and Olson (1943), and by Danish investigators, such as Winther and Hanson (1943), Siet & Jorgensen (1944), Bruhn (1944-1948) and Christiansen (1948). The efficiency of the test for both the detection of infected herds and the determination of infective lactating cows has been confirmed by Siet & Jorgensen (1944) and by Christiansen (1948). Those authors compared the results of the milk ring test with those of serum agglutination and, in respect of 16,000 herds, Christiansen (1948) found the two to be in accord in 93-95 per cent of instances. Again in comparison with the serum agglutination test, Roepke et al. (1949) reported that the milk ring test proved to be 75 per cent effectual in the discovery of reacting herds in Minnesota.

Minnesota./

The application of the milk ring test to the present investigation was intended primarily to screen milk samples for the presence of Brucella agglutinins and, incidentally, to permit comparison of the results with those of biological and cultural examinations. To carry out the test, 1.5 ml. of a well shaken sample of milk was placed into a narrow test-tube to form a column, approximately, 2.5 cm. high. One drop (0.04 ml.) of a well-shaken haematoxylinφ-stained suspension of Br. abortus antigen (Central Veterinary Laboratory Standard) was then added by means of a capillary glass pipette. After the contents had been thoroughly mixed by gentle inversion several times, the tube was incubated at 37°C. for one hour when the test was read. Results were recorded and interpreted as follows:

(a) + + + + (= Strongly positive) if the colour of the superficial cream layer was distinctly blue and that of the subjacent milk was white.

(b) + + + (= Positive) if the colour of the cream ring was distinctly blue and that of the rest of milk was faintly blue.

(c) + + (= Weakly positive) if the colour of the cream ring was distinctly blue and that of the milk also was blue.

(d) + (= Doubtful) if the blue colour of the cream ring was slightly deeper than that of the rest of the milk or if the contents of the tube were of uniform shade.

shade./

(e) - (= Negative) if the colour of the cream ring was white or slightly bluish and that of the rest of the milk remained distinctly blue, (fig. No. 3). In respect of the final group of samples, whilst the test continued to be used in the manner described above, the following modifications were introduced:

(1) Incubation at 37°C. was discontinued and, instead, the test-tube with its contents was left at room temperature for 5 minutes whereupon it was subjected to centrifugation at a maximal speed of 100 r.p.m. for 3 minutes. The results so obtained proved to be exactly the same as those given by the customary method.

(2) The material under test was left at room temperature for 5 minutes and was then centrifuged at 3000 r.p.m. for 3 minutes. When the results of this modified method came to be collated with those of the standard procedure, the following differences were observed:

(a) In the case of samples, which were strongly positive or positive by the standard procedure, the colour of the cream layer was distinctly blue whilst that of the milk was white.

(b) Samples, that were weakly positive by the standard test, exhibited two cream layers, the topmost of which was pale blue whereas the lower stratum was intensely blue and thinner whilst the subjacent milk was white and some of the stained antigen had settled on the bottom of the tube.

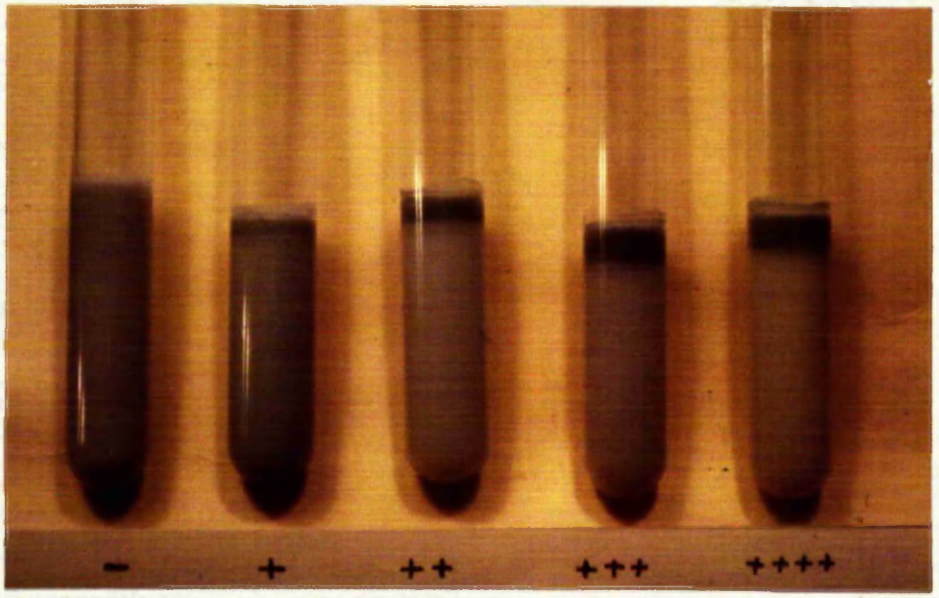


Fig. 3: Five different reactions obtained with the milk ring test after incubation at 37°C. for one hour.

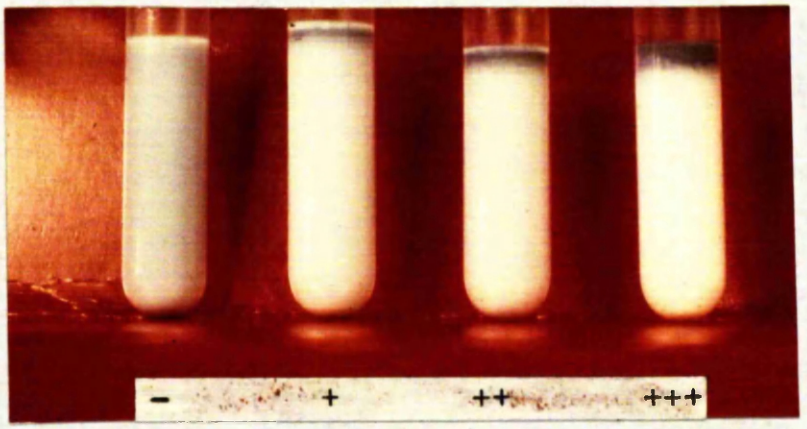


Fig. 4: Four different reactions obtained with the milk ring test carried out with the rapid centrifugation at 3000 r.p.m. (ca.) for 3 minutes.

tube./

(c) In the instance of samples, that gave a doubtful reaction by the customary ring method, the cream again occurred in two layers, the upper of which was white whilst the lower was blue and also very thin; the underlying milk was faintly bluish and the amount of antigen at the bottom of the tube exceeded that found in the preceding, or weakly positive, group. Indeed, some doubtful reactions fell into the negative group.

(d) Negative samples presented a single white layer of cream that rested upon milk which was blue in colour, (Fig. 4).

When test material was centrifuged for longer than 3 minutes, the appearance of the cream layer remained unaltered although, in the case of doubtful or negative samples, the milk was distinctly white and the sediment was more abundant. In all cases, the use of an angle centrifuge led to sharper results, as is clear if Figure 4 be compared with Figure 5. Indeed, some samples, that gave a doubtful reaction by the standard test, were distinctly negative after centrifugation at high speed.

2. THE MILK PLATE AGGLUTINATION TEST.

Diagnosis of brucellosis by means of the rapid microscopic serum agglutination test was first successfully demonstrated by Gwatkin (1923), the technique of whose test was subsequently improved by Huddleson & Abell (1928a). Gerber (1935) applied the procedure to

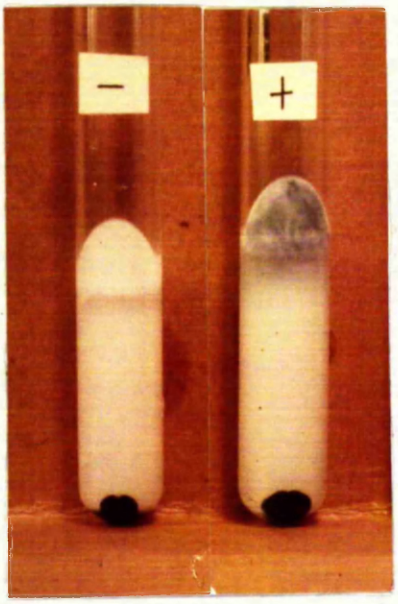


Fig. 5. Negative and doubtful reactions resulted by means of an angle centrifugation at 3000 r.p.m.

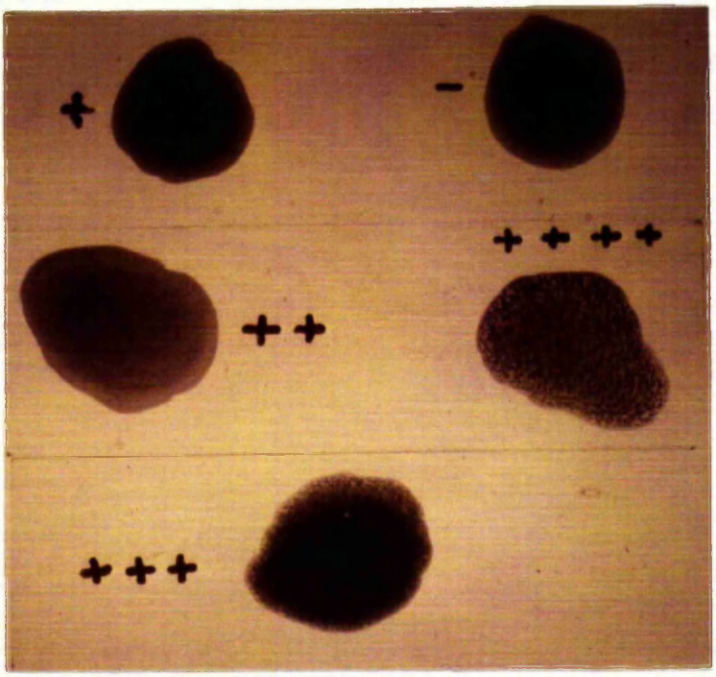


Fig. 6. Five different reactions given by the milk plate agglutination test after 12 minutes at room temperature.

to/

Brucella agglutinins in milk with the aid of a 20 per cent suspension of homologous bacteria which, he proposed, should be suitably stained. Finally, Blake et al.(1952) made use of the antigen prescribed for the milk ring test by mixing it with milk on a glass slide and reading the reaction after a lapse of 12 minutes at room temperature. Blake and his co- workers so tested 61 bovine and 122 caprine samples of milk and, after comparing the results with those of the serum agglutination, pronounced the plate method to have identified 99.7 per cent of infected cows. They stated that the best results were forthcoming by use of The Bureau of Animal Industry Antigen at a cellular concentration of 3 per cent and that a positive reaction was to be had when milk of an infected cow was diluted to as much as 1 in 20. A working party of the Public Health Laboratory Service (1956) reported that the milk plate test was not so sensitive as the milk ring reaction for identification of Brucella - infected herds after the results of both tests had been compared with those of biological examination in respect of 2,923 milk samples. Milunovic (1957) compared the milk plate agglutination test with the serum agglutination test in application to 186 cows from three healthy herds and to 270 cows from three herds afflicted by chronic brucellosis and concluded that the former method was a convenient and reliable one for routine screening of suspected herds. By means of the milk plate procedure together with the milk

milk/

ring test, McDiarmid et al (1958) investigated the state of immunity of individual cattle which had been immunised with strain 19 vaccine before breeding age and were subsequently exposed to infection by Brucella abortus. Those workers stated that both tests were of little value for their particular purpose, since milk from non-infected cows sometimes gave positive results whilst many samples from known infected animals proved negative to both reactions.

For milk plate agglutination, the writer adopted the following procedure. By means of a capillary glass pipette, 0.09 ml. (=three drops) of milk and 0.03 ml. (= one drop) of stained antigen were deposited on a scrupulously cleaned slide, mixed thoroughly with the aid of a bacteriological loop and left on the bench for 12 minutes under suitable cover. The result was read with bright light from a source located below the specimen and so arranged that only the mixture is illuminated. Any lateral light serves to obscure the small flocules that are encountered. Results, and the interpretation thereof, were reggraded as follows:

(a) + + + + (= Strongly positive), if about 75 per cent, or more, of the stained antigen was agglutinated into large flocules that were visible to the naked eye.

(b) + + + (= Positive), if from 50-75 per cent of the stained antigen was agglutinated.

agglutinated./

(c) + + (= Weakly positive), if less than 50 per cent, but more than 25 per cent, of the stained antigen was agglutinated.

(d) + (= Doubtful), if less than 25 per cent of the antigen was agglutinated or if only a few small flocules were appreciable.

(e) - (= Negative), if signs of agglutination were not perceptible and the mixture remained homogenously opaque (Fig. 6).

3. THE MILK CAPILLARY TUBE TEST.

The capillary tube test was first introduced by King (1951) for the detection of Brucella agglutinins in milk and for the diagnosis of brucellosis in infected lactating cows. The procedure is also based on the phenomenon of agglutination and the requisite antigen is same as that used in the milk ring test. Antigen is brought into contact with the milk under test in a capillary glass tube. In the case of a positive reaction agglutination of the stained antigen gives rise to large flocules which by means of bright light, are seen to be scattered throughout the milk. The reading of the test is facilitated by use of a hand lens.

To perform the test, one end of a capillary tube, 8 cm. long, is immersed into the stained antigen where it is held until fluid rises to a height of, approximately, 5-6 millimetres. Any excess of suspension may be removed if the tip of the tube be lightly touched with filter-paper. If the antigen does not readily ascend, rotation

rotation/

of the tube between the forefinger and the thumb may prove helpful but should the inside of the capillary tube be wet, the fluid will mount quickly. The tip of the tube is then introduced at an acute angle into the sample of milk under test when the latter proceeds to rise and mixes with the antigen to yield an uniformly blue column. As soon as the fluid approximates the upper end of the capillary tube, the latter is closed by application of the forefinger and, held in the horizontal position, it is transferred to a piece of plasticine. Therein the lower end is inserted so that the tube rests at an angle of 45°, and the reaction allowed to proceed for thirty minutes at room temperature ere it is read.

Despite the fact that it is easy to perform and is practicable under field conditions, the capillary tube test has not yet been used as much as has the milk ring reaction. It has been employed, however, in America by Morse & Pope (1952), in Britain, by a working party set up by the Public Health Laboratory Service (1956) and also by Jameson (1957). The value of the test for the detection of Brucella agglutinins in milk was confirmed by those workers. King (1951) reported, after examination of 428 samples from individual animals, an agreement of 92.7 per cent between the capillary method and the blood serum agglutination test. Morse & Pope (1952) found the capillary method to be useful for the screening of quarter milk samples submitted

submitted/

for cultural evidence of Br. abortus and recovered the micro-organism from 171 samples of milk which gave a strongly positive reaction and from 2 that were positive. They were unsuccessful in their attempts to isolate Brucellae from samples that proved negative to or were doubtful by capillary tube test.

In the writer's experience, the changes encountered in the capillary tube test, performed as described above, and the relevant interpretations may be summarized, thus:

(a) + + + + (= Strongly positive), when the column was found to consist of white milky parts, separated by deep blue portions comprised of clumps of agglutinated antigen.

(b) + + + (= Positive), when there occurred a mixture of bluish-white milk and antigen together with deep blue flocculi, consisting of agglutinated bacilli.

(c) + + (= Weakly positive), when the column appeared bluish throughout with several aggregates of deep blue colour.

(d) + (= Doubtful), when a few clumps were found scattered widely throughout a distinctly blue column.

(e) - (= Negative), when the column of milk and antigen was uniformly blue in colour and the lower part of the capillary tube presented a continuous thin blue line along most of its length, (Figs. 7 and 8).

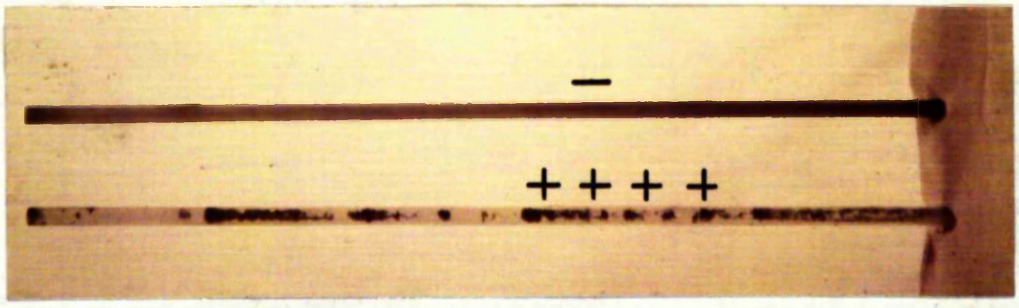


Fig. 7. A positive and a negative reaction given by the capillary tube test after 30 minutes at room temperature.

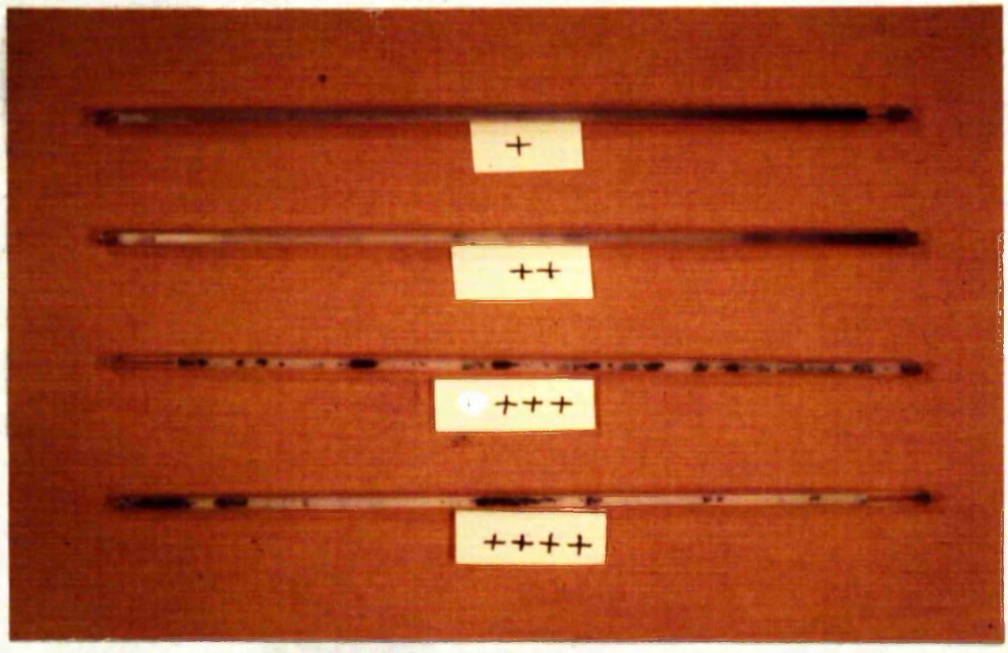


Fig. 8. Four different positive reactions obtained by the capillary tube after 30 minutes at room temperature.

(Figs. 7 and 8)./

4. THE MILK WHEY-TUBE AGGLUTINATION TEST.

Zammit (1906) was the first to observe that, for diagnostic purposes, an agglutination test could be applied to the milk of goats affected by Malta fever as well as to blood-serum. The presence of Br. abortus-specific agglutinins in milk was also mentioned by McFadyean & Stockman (1909). Kennedy (1914) demonstrated the presence of Brucella agglutinins in 5 out of 13 samples of cow's milk.

Seddon (1915) suggested that, in certain cases, milk might satisfactorily replace blood-serum in the agglutination test although it had been previously deemed unsuitable on account of its physical properties. To overcome that difficulty, he first clotted the milk by means of lactic acid. Finally, the presence of Br. abortus agglutinins in bovine milk was demonstrated by Cooledge (1916). Later, Little & Orcutt (1922) used rennet to clot samples of milk destined for serological tests. Those authors also showed that Br. abortus agglutinins pass from mother to calf via the milk.

The physical difficulty of obtaining blood samples in certain areas together with the necessity for frequent testing of herds afflicted by a high incidence of the disease constituted reason enough for the investigators to use whey for the detection of the presence of agglutinins in the animal body. A direct correlation appeared to exist between the amount of antibody present in blood and that in milk

milk/

and further research proved the whey agglutination test to be a valuable one. Smith et al.(1923) showed that the ratio of Brucella agglutinins in the milk to those in the blood of infected cows varied from 1/32 to 1/64 and that, when Br. abortus became localised in the udder, the amount of specific agglutinin in the milk rose whereby the ratio was altered to 1/4, 1/2 or even beyond 1/1.

For the diagnosis of Bang's disease, Huddleson & Abell (1928b) described a rapid microscopic serum agglutination test, which technique was recommended by Torrey (1929) as applicable to whey for the purpose of detecting cows with infected udders. Graham & Thorp (1930) compared the standard whey tube agglutination test with Huddleson's rapid method in relation to, approximately, 464 of bovine milk samples. The sensitivity of the two procedures was almost equal butthe rapid test was attended by a greater number of doubtful reactions. Both methods of whey agglutination are now used for the detection of Brucella antibody in milk.

Karsten (1931) stated that the amount of antibody in serum was about ten times greater than that detectable in whey. He reported that in only two out of 31 instances in which the serum agglutination test was positive did he find the whey agglutination test to be negative. Montgomerie & Rowlands (1932) stated that the whey test might with advantage be more frequently employed. Diernhoper (1933) and Hall &

Hall &/

Learmonth (1933) used rennet and chloroform to obtain clear whey from cow's milk.

Reidmuller (1934) declared that the rapid whey agglutination test usually gave positive results in the case of milk samples obtained from cows with a blood titre of 1/10 and upward. In a survey of 469 samples of milk, he found that in 13 instances the rapid test failed to detect infection in animals in which titres of 1 in 10 or 1 in 20 had been determined by means of the standard tube test.

In the investigation reported in this thesis, whey was obtained by adding three drops of rennet and about 0.5 ml. of chloroform to 2.3 ml. of milk in a test tube. The mixture was then thoroughly shaken so that the chloroform became emulsified and served to dissolve fat-globules. After incubation at 37°C. for one hour, the mixture was centrifuged and the clear supernatant whey employed for the test.

The Brucella antigen adopted for the experiment was supplied by the Central Veterinary Laboratory Weybridge and, before use, was diluted to 1 in 10 with sterile normal (0.85 per cent) saline solution.

The test was performed in narrow (75 mm. x 8 mm.) test-tubes, five of which were set up in a wooden rack. By means of a graduated pipette, of one millilitre capacity and calibrated to the tip, 0.8 ml. of normal saline solution was transferred into the first tube of the

the/

series and 0.5 ml. into the remaining four tubes. 0.2 ml. of whey was added to the first tube and thoroughly mixed with the saline, so to provide a whey dilution of 1/5. 0.5 ml. of the latter was removed into the second tube and the contents mixed to yield a dilution of 1/10. The procedure was continued in the case of the other tubes but from the last one 0.5 ml. of fluid was discarded in order that each tube of the series should contain 0.5 ml. of whey diluted to 1/5, 1/10, 1/20, 1/40, and 1/80, respectively. To each tube was then added 0.5 ml. of standard Brucella antigen whereby final dilutions of 1/10, 1/20, 1/40, 1/80, and 1/160 were forthcoming. The test was read after incubation at 37°C. for 24 hours.

As the experiment progressed, it came to be realised that additional whey dilutions, of the order of 1/2, 1/4, 1/8 et al., promoted the detection of the small amounts of agglutinin that may occur in milk. Accordingly, the procedure was modified to provide for dilutions of whey that ranged from 1 in 2 to 1 in 160.

B. BIOLOGICAL EXAMINATION.

In the case of the first and the second groups, about 120 ml. of a well-shaken sample of milk was distributed into four wide-mouth bottles, of 1 oz. capacity, and centrifuged at 3,000 r.p.m. for 30 minutes. By means of a pipette, the separated milk was withdrawn save for about one millilitre, in which the cream and the sediment

sediment/

were suspended and 2-3 ml. of the mixture was then injected intramuscularly into the thigh of a guinea-pig.

In the instance of the third and the fourth groups of samples, the amount of milk employed lay between 10 and 25 ml. Each sample that had proved positive to or was indeterminate by serological tests was usually injected into one guinea-pig but, occasionally, two animals were used. In the case of serologically negative samples, the sediment after centrifugation and the cream of two or three of them were suspended individually in a millilitre of separated milk and then mixed together. Three ml. of the mixture was then inoculated intramuscularly into one guinea-pig, 1.5 ml. being injected into one thigh and the remainder into the opposite limb. Occasionally, one guinea-pig was used for a particular sample.

After inoculation, guinea-pigs were isolated in individual cages where they were kept under observation for a maximal period of eight weeks. If an abscess developed at the site of inoculation, it was lanced whilst it was soft and the pus expressed. Thereafter, the open wound was disinfected with iodine or was treated with sulphamethazine. Three or four weeks after injection, the guinea-pig was bled from the heart to provide serum that was subjected to the agglutination test for Brucella infection. If a reaction to a titre beyond 1 in 160 were so forthcoming, the guinea-pig was sacrificed.

sacrificed./

Animals with a lower titre were retained until the end of the sixth week after inoculation. Kept for a period of eight weeks were those creatures which had been inoculated with serologically positive or serologically doubtful milk and which had failed to develop demonstrable serum-agglutinins in three to four weeks after injection. Finally, if the guinea-pig had been inoculated with a serologically negative sample and three or four weeks later was found not to contain agglutinins in the blood serum, it was sacrificed seven weeks after injection. Apart from those destroyed at the end of the third or fourth week of infection, all of the guinea-pigs were bled again before they were killed and the serum agglutination test was again carried out. Immediately after it was bled, a guinea-pig was destroyed by dislocation of the neck and subjected to autopsy, during which cultures were made from the spleen and from any enlarged inguinal or axillary lymph-nodes (Fig. 9) that were encountered.

Films from affected organs were stained by Koster's method in which, after fixation by heat, the preparation is treated with dilute carbol-fuchsin (1 in 10 in distilled water) for 5 to 7 minutes, washed with distilled water and decolorized with 0.5 per cent acetic acid when it is again washed with distilled water. The preparation is then counterstained with Loeffler's methylene-blue for 45 seconds and washed with distilled water before it is dried by

Fig. 9: A normal (right) and a large infected spleen (left) from guinea-pigs and related inguinal lymphatic nodes.

by/

blotting and in the air. By Koster's technique, Brucella organisms are coloured red and most other organisms as well as tissue cells are stained blue. In films from some of the serologically positive cavies, a few Brucella like micro-organisms were visible. Attempts to take a convincing photomicrograph were largely unsuccessful, (Fig. 10 is one of the more satisfactory of those efforts, and is available for comparison with Fig. 11 which is presented on the same page.) The tissues were stored in a refrigerator until the results of cultural examinations became available. For primary isolation, the surface of an organ was seared by means of a spatula that had been heated to redness in a Bunsen flame and through that area was inserted a capillary glass pipette, by means of which fluid or a fragment of tissue was extracted and inoculated either to blood agar, or to serum dextrose agar, as used by Jones & Morgan (1958). The plates were then incubated at 37°C. for 3 to 15 days inside a jar made to contain about 10 per cent CO_2. Throughout the period of incubation, the plates were examined every third day, and visible colonies were studied morphologically and by means of films stained by Gram's method. If any resemblance to Brucella micro-organisms was detectable, a colony was subcultivated and the resulting micro-organisms examined by both the rapid and the tube agglutination tests with the aid of polyvalent Brucella antiserum.

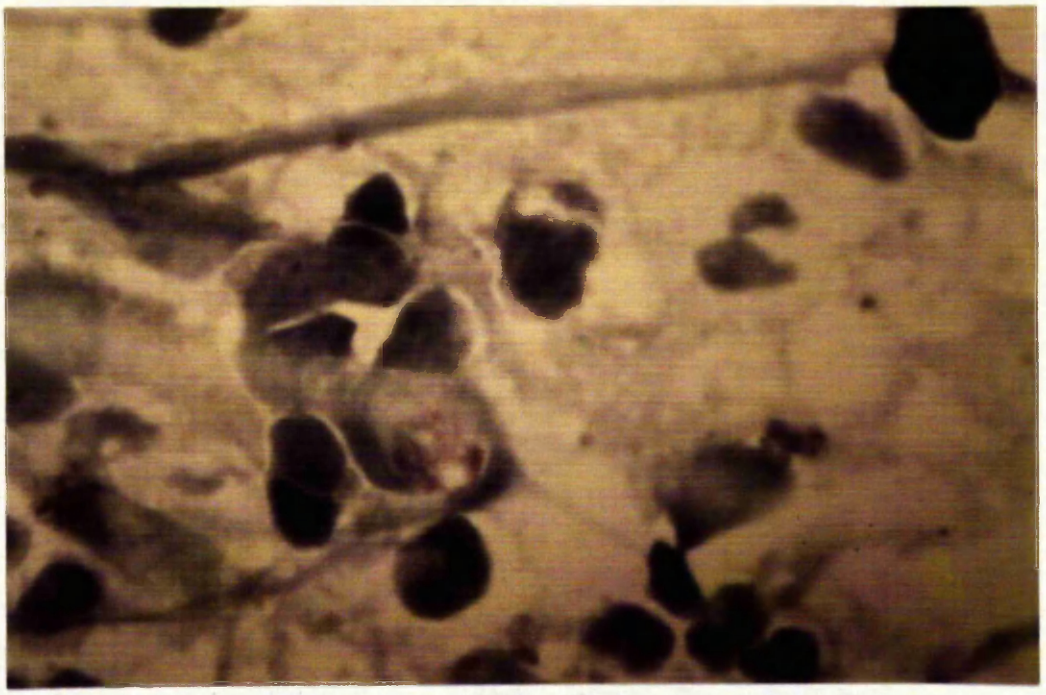


Fig. 10: Smear from a Brucella-infected spleen stained by Koster's method (x 1330).

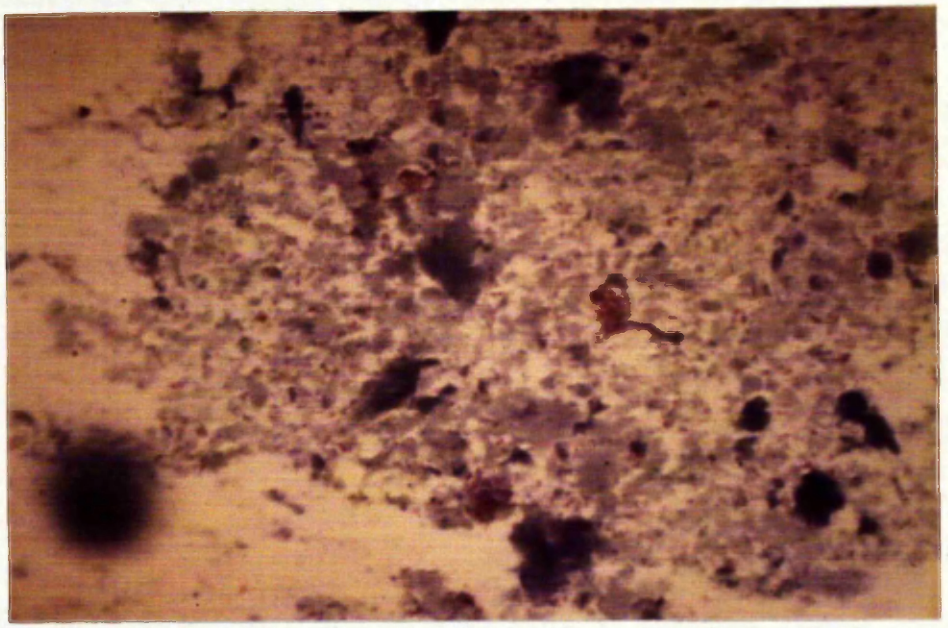


Fig. 11: Film of stomach contents of an aborted bovine foetus, stained by Koster's method (x 1330).

antiserum./

Brucella cultures so isolated were ultimately identified not only serologically but also biochemically and by means of phage typing.

The guinea-pigs chosen for experiment were generally not less than 600 grams in weight in order that they might better tolerate the massive injections involved. When creatures of lesser weight had to be used the inoculum was halved and one portion was inoculated into each of two guinea-pigs.

Before injection, the guinea-pigs were not subjected to serological examination for Brucella infection, for the reason that they had been bred in relative isolation in the Veterinary School and beyond contact with the usual sources of Brucella infection. If some risk was thereby incurred, it did not prove serious, at any rate, in the light of the results forthcoming from the experiments with the fifty samples of pasteurised milk.

C. CULTURAL EXAMINATION.

The development of a medium suitable for the isolation of Brucella micro-organisms from the milk of infected animals was a problem that arose as soon as Horrocks (1905) and Schroeder & Cotton (1911) proved that Br. melitensis and Br. abortus were present in the milk of infected goats and cows. Evans (1915, 1916 and 1918) was the first to isolate Br. abortus by cultivation from individual milk samples obtained hygienically from infected cows. She used 10 per cent bovine serum

serum/

agar as a medium for growth but found that it did not favour the development of Brucella micro-organisms from every sort of milk and was least suited for samples of bulk milk, largely, on account of the presence of rapidly growing contaminants. Hasley (1930) resorted to liver infusion agar containing 1/200,000 gentian violet for the isolation of Br. abortus from samples of certified milk. Stocknayer (1933) employed liver infusion agar containing brilliant green or gentian violet, both to a concentration of 1/250,000. The two latter media remained in use until Felsenfeld et al. (1951) devised one consisting tryptose agar fortified by 10 units of Circulin, 10 units of Polymyxin D, 25 units of Bacitracin and 230 micrograms of Sulphadiazine per millilitre. The authors stated that their medium enabled Brucella to be recovered in pure culture from chicken faeces. Kusdas & Morse (1953) described another medium containing albimi-agar supplemented by 6,000 units of Polymyxin B. Sulphate, 100 mg. Actidione, 25,000 units Bacitracin, 15,000 units Circulin and 1.4 mg. Crystal violet per litre. Morris (1956) described still another medium composed of Tryptose agar plus 1/3,000 (v/v) of 5-nitrofurfurylmethyl ether, 1/10,000 (w/v) Actidione, 25 units of Bacitracin and 4 units of Polymixin B. per millilitre. Morris claimed that micro-organisms normally to be encountered in cultures from faeces or soil were completely suppressed on that medium whereas Brucella grew abundantly within 65 hours of

of/

incubation.

Because certain fastidious types of Br. abortus, such as Type 2 Wilson, do not grow on commercial media, e.g. albimi-agar, tryptose agar and trypticase soy agar without the addition of serum (Jones & Morgans 1958), and because they do not develop in the presence of bacteriostatic dyes (Huddleson & White 1954), Morgan (1960) compared nine different media and concluded that serum-dextrose-agar plus antibiotics (S.D.A. medium) was the only selective medium which supported the growth of all the Brucella cultures studied. The antibiotics used were 100 mg, Actidione,6,000 units Polymyxin B. and 25,000 units of Bacitracin per litre.

Williams et al. (1962) demonstrated a direct relationship between the presence of Erythritol in the Foetal tissues and the massive localization of Br. abortus in the foetus. They showed that Erythritol was a normal product of the foetal tissues of cows and certain other animals and that in the cotyledons, in which that Alcohol occurred to high concentration, colonization by Br. abortus was heavier. They also proved that Erythritol stimulated the growth of Br. abortus in vitro. Such findings suggest that the addition of Erythritol to selective media may promote the recovery of Br. abortus from milk and other contaminated material.

The media which were used throughout this experiment were as follows.

follows./

(1) Five per cent horse blood agar (2) Serum dextrose agar (S.D. medium) and Serum dextrose agar plus antibiotics (S.D.A. medium). The two latter media were described by Jones & Morgan (1938) who claimed that S.D.A. medium was suitable for the selective isolation of Brucella micro-organisms from milk and other contaminated substances.

S.D. and S.D.A. media were applied to cultivation from samples of milk. S.D. medium and, more often horse blood agar, were used for the subcultivation of colonies resembling those of Brucella. For the preparation of S.D.A. medium Bacitracin was obtained from Glaxo Laboratories, Ltd., and was found to suppress the growth of all standard strains of Br. abortus, Br. melitensis and Br. suis. It was therefore, omitted and the experiments proceeded with a medium that contained only Polymyxin B. and Actidione, to the concentrations mentioned above.

For the first, second and third groups of milk samples four plates of S.D. and four plates of S.D.A. were inoculated. One drop of a mixture of the sediment and cream, that subserved the biological test, was deposited on the surface of the plate to one side where it was spread over a small area by means of a sterile loop. The latter was again sterilised and then used to spread the inoculum thinly over the remaining expanse of plate. Two plates of S. D. medium and two of S.D.A. medium were placed in a McIntosh and Fildes jar together with a test-tube containing some marble chips and a few millilitres of hydrochloric acid diluted 1 in 4 (v/v) with distilled water.

water./

(The increased tension of carbon dioxide gas so produced sufficed to promote the growth of Br. abortus.) The jar was then closed and incubated at 37°C. for 3 to 12 days. Two other plates of S. D. medium and two of S.D.A. medium were simultaneously incubated under aerobic conditions. In the case of the fourth group of milk samples the four plates of S.D.A. medium were all incubated in an atmosphere containing added carbon dioxide.

The plates were studied every 3 days and any suspicious colonies were subcultivated to either blood-agar or S. D. medium. For subcultivation, each plate of medium was divided into four sections and each section was inoculated from a different colony, the origin of which was marked by means of a wax pencil. The plates were incubated under the conditions that had applied to those from which they arose. After 3 to 6 days the cultures were examined by means of films stained by Gram's method. Cultures of organisms morphologically resembling Brucella were then subjected to both the plate (or rapid) and the tube (or slow) methods of agglutination with antiserum either supplied by the Wellcome Research Laboratories, Beckenham or prepared by the writer from known Brucella infected guinea-pigs. When positive results were so obtained, the cultures were subjected to further serological and biochemical tests as well as to phage typing.

typing./

D. MICROSCOPICAL EXAMINATION.

Szalay (1942) had reported the demonstration of Brucellae in milk by microscopal examination of films stained by the method described by Koslowsky.

During the work under record, microscopy was applied to mixtures of the sediment and cream obtained after centrifugation of the first group of milk samples, the requisite films having been stained by Koster's method. For allthe specimens in the second, third and fourth groups, microscopical examination was discontinued because it proved to be of little value in the detection of Brucellae in milk.

4. RESULTS.

The information tabulated on pages 64 to 70 has been abstracted from the mass of results which accrued from the biological and various serological examinations that were applied to 349 samples of milk that were of the following origin:

(1) 114 samples of certified milk (1st. group: Table 1, page 64);

(2) 78 samples of bulk raw milk (2nd. group: Table 2, page 66);

(3) 50 samples of the preceding milk after it had been subjected to pasteurisation (2nd. group: Table 3, page 67);

(4) 56 samples of milk procured from cows which had aborted from various causes (3rd. group: Table 4, page 68) and

and/

(5) 51 samples of milk from cows of an attested herd, to which latter a case of human brucellosis had been traced (4th group: Table 5, page 70).

Tables 1A (page 65) and 4A (page 69) are extensions of tables 1 and 4. The former gives the results of 14 repeat samples of the first group and the latter reveals the results of 23 teat samples from 6 animals in the third group.

Here, it is necessary to emphasise that tables 1-5 have been simplified by omission of all the samples of milk which proved to be wholly negative, that is, which did not respond to any of the chosen procedures of study. In that way, much tedious repetition has been obviated without, it is claimed, any sacrifice of essential data.

In the case of cultural examination, Brucellae were recovered directly from only five samples of milk, of which one pertained to group one, another was derived from group 3 and the remaining three belonged to group four. Those samples were also positive with the biological procedure.

Microscopical examinations in the first group gave inconclusive results only in two instances in which the results of the biological procedure proved to be positive.

Results of the standard ring test in the fourth group of samples are available in Table 5A (Page 71) for comparison with those forthcoming when that test was modified by centrifugation at slow

slow/

and at high speeds.

Tables 6 - 10 (pages 72 to 74) also summarise the percentage of findings obtained with the different groups of samples tested by the four serological tests employed.

Table 13 (page 77) is presented diagrammatically of all the results obtained with the four groups of samples. On the left side are given the groups, the number of the milk samples and the number of the farms and animals concerned. In the middle part, the results of screening by the six methods of testing are tabulated and the number and nature of the various reactions are recorded. Finally, on the right side of the diagram, the incidence of Brucella infection encountered in the four groups of milk are stated.

Fig. 11 which succeeds table 13, gives the geographical location of those herds from which Brucella micro-organisms were isolated.

TABLE 1.

Sample No.	Date Collected.	CERTIFIED MILK.						
		Direct Investigation.				Biological Examination.		
		Serological Examination.				Serological Tests.		Culture ex organs
		M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st. Bleed.	2nd. Bleed.	
5	10. 2.61	+	Neg.	Neg.	Neg.	1/40	1/1280	Pos.
7	14. 2.61	++++	+++	+++	1/10	Neg.	Neg.	Neg.
12	21. 2.61	++++	++++	++++	1/20	1/20	1/640	Pos.
15	28. 2.61	++++	++++	++++	1/40	1/40	1/160	Pos.
19	7. 3.61	++++	++++	++++	1/160	1/80	1/1280	Pos.
22	14. 3.61	+++	+++	+++	Neg.	Neg.	Neg.	Neg.
43	21. 4.61	+++	+++	+++	Neg.	Neg.	1/320	Pos.
45	25. 4.61	+++	+++	+++	Neg.	Neg.	Neg.	Neg.
46	25. 4.61	Neg.	Neg.	Neg.	Neg.	Neg.	1/640	Pos.
52	5. 5.61	+	+	+	Neg.	Neg.	Neg.	Neg.
53	5. 5.61	++	++	++	Neg.	Neg.	Neg.	Neg.
60	16. 5.61	++	+	+	Neg.	Neg.	Neg.	Neg.
69	3. 6.61	++++	++++	+++	1/20	Neg.	Neg.	Neg.
77	9. 6.61	++++	++	+++	Neg.	1/80	1/320	Pos.
84	16. 6.61	++++	++++	++++	1/20	Neg.	1/160	Pos.
87	20. 6.61	++++	+++	+++	1/10	1/20	1/320	Pos.
89	14. 6.61	+++	++	+++	Neg.	Neg.	Neg.	Neg.
93	27. 6.61	+	+	Neg.	Neg.	Neg.	Neg.	Neg.
97	5. 7.61	+	+	+	Neg.	Neg.	Neg.	Neg.
101	7. 7.61	+++	++	++	Neg.	Neg.	Neg.	Pos.
108	18. 7.61	++++	++++	++++	1/20	1/20	1/1280	Pos.
112	21. 7.61	+	Neg.	+	Neg.	N.T.	1/1280	Pos.
113	21. 7.61	+++	+	+	Neg.	1/40	1/2560	Pos.
114	31. 7.61	+++	++	++	Neg.	Neg.	1/20	Neg.

M.R.T. = Milk ring test.

M.P.T. = Milk plate test.

M.C.T. = Milk Capillary tube test.

M.W.T. = Milk Whey agglutination test.

Acrob. = Aerobically.

Bleed. = Bleeding.

Neg. = Negative.

N.T. = Not tested.

\+ = Indicates the nature of positive reaction.

T A B L E 1A

Sample No.	Date Collected	REPEAT SAMPLES OF CERTIFIED MILK						
		DIRECT INVESTIGATION				BIOLOGICAL EXAMINATION		
		SEROLOGICAL EXAMINATION.				SEROLOGICAL TESTS		Culture
		M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st. Bleeding	2nd. Bleeding.	ex organs.
7	18.4.61	++++	++++	++++	1/10	1/640	1/640	Pos.
45	19.5.61	+++	++	++	Neg.	1/20	1/40	Pos.
52	26.9.61	+	+	Neg.	Neg.	Neg.	Neg.	Neg.
53	26.9.61	+	+	Neg.	1/2	Neg.	Neg.	Neg.
60	29.9.61	++	+	+	1/2	Neg.	Neg.	Neg.
69	22.9.61	++++	+++	+++	1/20	Neg.	Neg.	Pos.
93	22.9.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
97	31.7.61	++	+	+	Neg.	1/60	N.T.	Pos.

M.R.T. = Milk ring test.

M.P.T. = Milk plate test.

M.C.T. = Milk capillary tube test.

M.W.T. = Milk whey agglutination test.

Neg. = Negative.

N.T. = Not tested.

\+ = Indicate the nature of positive reactions.

TABLE 2.

Sample No.	Date Collected.	BULK RAW MILK						
		DIRECT INVESTIGATION				BIOLOGICAL EXAMINATION		
		SEROLOGICAL EXAMINATION.				SEROLOGICAL TESTS		Culture ex organs.
		M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st. Bleeding.	2nd. Bleeding.	
1R	27. 7.61	++	++	+	Neg.	Neg.	Neg.	Neg.
2R	27. 7.61	+++	++	++	Neg.	Neg.	Neg.	Neg.
3R	4. 8.61	++++	++	++	Neg.	1/160	N.T.	Pos.
5R	8. 8.61	++	++	+	Neg.	Neg.	Neg.	Pos.
6R	8. 8.61	+	+	Neg.	Neg.	Neg.	Neg.	Neg.
7R	8. 8.61	+++	++	+	Neg.	Neg.	Neg.	Neg.
10R	15. 8.61	Neg.	Neg.	Neg.	Neg.	Neg.	1/20	Pos.
11R	15. 8.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
12R	15. 8.61	+++	++	++	1/8	Neg.	Neg.	Neg.
13R	18. 8.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
14R	18. 8.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
15R	6.10.61	++++	++	++	1/4	1/40	1/320	Pos.
16R	6.10.61	++	+	Neg.	1/2	1/10	1/160	Pos.
17R	6.10.61	++	+	+	1/2	Neg.	Neg.	Neg.
18R	6.10.61	++	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
20R	10.10.61	++	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
23R	12.10.61	+++	Neg.	+	1/4	Neg.	Neg.	Neg.
24R	12.10.61	++	+	++	1/8	Neg.	Neg.	Neg.
25R	16.10.61	+++	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
26R	16.10.61	+++	Neg.	Neg.	1/4	Neg.	Neg.	Neg.
27R	16.10.61	+++	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
28R	16.10.61	+++	+	+	1/4	Neg.	Neg.	Neg.
29R	20.10.61	++	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
30R	20.10.61	++	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
34R	31.10.61	++	Neg.	+	Neg.	Neg.	Neg.	Neg.
36R	3.11.61	++	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
37R	3.11.61	+++	++	+	Neg.	Neg.	Neg.	Neg.
38R	3.11.61	++++	++++	++++	1/40	1/160	1/640	Pos.
39R	3.11.61	Neg.	Neg.	Neg.	Neg.	1/40	1/320	Pos.
41R	13.11.61	++	Neg.	Neg.	1/4	Neg.	Neg.	Neg.
45R	21.11.61	+++	Neg.	Neg.	Neg.	1/80	1/320	Pos.
46R	22.11.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
47R	22.11.61	++++	++	+++	Neg.	Neg.	Neg.	Neg.
48R	22.11.61	++++	++	+++	Neg.	Neg.	Neg.	Neg.
49R	22.11.61	++++	+	Neg.	Neg.	Neg.	Neg.	Neg.
50R	22.11.61	++	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
61R	14.12.61	++	Neg.	+	Neg.	Neg.	Neg.	Neg.
62R	14.12.61	+++	Neg.	+	Neg.	Neg.	Neg.	Neg.
67R	15.12.61	++	Neg.	+	Neg.	Neg.	Neg.	Neg.
69R	15.12.61	+++	+	+	Neg.	Neg.	Neg.	Neg.
78R	20.12.61	++++	+++	++	Neg.	Neg.	Neg.	Neg.

M.R.T. = Milk ring test.

M.P.T. = Milk Plate test.

M.C.T. = Milk capillary tube test.

M.W.T. = Milk whey agglutination test.

Neg. = Negative.

N.T. = Not tested.

\+ = Indicate the nature of positive reaction.

TABLE 3. PASTEURIZED MILK.

Sample No.	Date Collected.	DIRECT INVESTIGATION				BIOLOGICAL EXAMINATION		
		SEROLOGICAL EXAMINATION				SEROLOGICAL TESTS.		Culture
		M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st. Bleeding.	2nd. Bleeding	ex organs
1P	27. 7.61	++	++	+	Neg.	Neg.	Neg.	Neg.
2P	27. 7.61	++	++	++	Neg.	Neg.	Neg.	Neg.
3P	4. 8.61	+++	++	++	Neg.	Neg.	Neg.	Neg.
5P	8. 8.61	+	++	+	Neg.	Neg.	Neg.	Neg.
6P	8. 8.61	+	+	Neg.	Neg.	Neg.	Neg.	Neg.
7P	8. 8.61	+	++	+	Neg.	Neg.	Neg.	Neg.
11P	15. 8.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
12P	15. 8.61	++	++	++	1/8	Neg.	Neg.	Neg.
15P	6.10.61	++	++	++	1/4	Neg.	Neg.	Neg.
16P	6.10.61	+	+	Neg.	1/2	Neg.	Neg.	Neg.
17P	6.10.61	+	+	+	1/2	Neg.	Neg.	Neg.
18P	6.10.61	+	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
20P	10.10.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
23P	12.10.61	++	Neg.	+	1/4	Neg.	Neg.	Neg.
24P	12.10.61	+	+	++	1/8	Neg.	Neg.	Neg.
25P	16.10.61	++	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
26P	16.10.61	+++	Neg.	Neg.	1/4	Neg.	Neg.	Neg.
27P	16.10.61	++	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
29P	20.10.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
30P	20.10.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
34P	21.10.61	Neg.	Neg.	+	Neg.	Neg.	Neg.	Neg.
36P	3.11.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
37P	3.11.61	++	++	+	Neg.	Neg.	Neg.	Neg.
41P	19.11.61	+	Neg.	Neg.	1/4	Neg.	Neg.	Neg.
45P	21.11.61	++	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
46P	22.11.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
47P	22.11.61	++	++	++	Neg.	Neg.	Neg.	Neg.
48P	22.11.61	++	++	++	Neg.	Neg.	Neg.	Neg.
49P	22.11.61	++	+	Neg.	Neg.	Neg.	Neg.	Neg.
50P	22.11.61	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

M.R.T. = Milk ring test.

M.P.T. = Milk plate test.

M.C.T. = Milk Capillary tube test.

M.W.T. = Milk whey agglutination test.

Neg. = Negative.

+ = Indicates the nature of positive reaction.

TABLE 4.

Sample No.	Date Collected.	MILK FROM ABORTING ANIMALS.						
		DIRECT INVESTIGATION				BIOLOGICAL EXAMINATION		
		SEROLOGICAL EXAMINATION.				SEROLOGICAL TESTS.		Culture ex organs.
		M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st. Bleeding	2nd. Bleeding.	
1A	7. 4.61	++++	+++	++++	1/40	Neg.	Neg.	Neg.
4A	18. 4.61	++++	++++	++++	1/40	Neg.	Neg.	Neg.
16A	6. 7.61	N.S.	++	+++	Neg.	Neg.	Neg.	Neg.
17A	6. 7.61	+	Neg.	++	Neg.	Neg.	Neg.	Neg.
18A	6. 7.61	Neg.	Neg.	+	Neg.	Neg.	Neg.	Neg.
19A	6. 7.61	++++	++++	++++	1/40	Neg.	Neg.	Neg.
22A	6. 7.61	++++	++++	++++	1/20	Neg.	Neg.	Neg.
29A	6. 7.61	++++	++++	++++	1/320	1/320	1/1280	Pos.
30A	6. 7.61	++++	++++	++++	1/160	1/80	1/320	Pos.
32A	15. 8.61	++++	++++	++++	1/20	Neg.	Neg.	Neg.
33A	3.10.61	++++	++++	++++	1/20	Neg.	Neg.	Neg.
34A	13.10.61	++++	++++	++++	1/80	1/10	1/80	Pos.
35A	25.10.61	+++	++	++	1/20	Neg.	1/80	Pos.
37A	25.10.61	++++	++++	++++	1/320	1/20	1/80	Pos.
38A	3.11.61	++++	++++	++++	1/320	1/80	1/1280	Pos.
39A	3.11.61	++++	++++	++++	1/640	Neg.	1/20	Pos.
40A	3.11.61	++++	++++	++++	1/1280	1/160	1/640	Pos.
41A	3.11.61	++++	++++	++++	1/160	1/40	1/640	Pos.
42A	13.11.61	++	++	++	1/80	Neg.	Neg.	Neg.
43A	29.11.61	++++	++	++++	1/40	Neg.	Neg.	Neg.
44A	17.11.61	+	Neg.	+	Neg.	Neg.	Neg.	Neg.
45A	4.12.61	+++	Neg.	+	Neg.	Neg.	Neg.	Neg.
46A	21.12.61	++++	+++	+++	Neg.	1/80	1/640	Pos.
47A	8. 1.62	Neg.	Neg.	Neg.	1/2	Neg.	Neg.	Neg.
49A	20. 1.62	++++	++++	++++	1/40	Neg.	Neg.	Neg.
52A	23. 2.62	N.S.	++++	++++	1/1280	1/640	N.T.	Pos.
53A	5. 2.62	++++	++++	++++	1/160	1/320	N.T.	Pos.
54A	13. 5.63	++++	++++	++++	1/320	1/640	N.T.	Pos.

M.R.T. = Milk ring test.

M.P.T. = Milk Plate test.

M.C.T. = Milk capillary test.

M.W.T. = Milk whey agglutination test.

Neg. = Negative.

Pos. = Positive.

N.S. = Not suitable for the test.

N.T. = Not tested.

TABLE 4. MILK RING TEST OF 6 ABORTED ANIMALS.

Sample No.	Test No.	Date Collected	DIRECT INVESTIGATION				SEROLOGICAL EXAMINATION		
			SEROLOGICAL EXAMINATION				SEROLOGICAL TESTS.		Culture ex organs.
			M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st.Bleeding	2nd.Bleeding	
4A	(1)	10. 4.61	++++	++++	++++	1/20	Neg.	Neg.	Neg.
"	(2)	" " "	++++	++++	++++	1/20	Neg.	Neg.	Neg.
"	(3)	" " "	++++	++++	++++	1/40	Neg.	Neg.	Neg.
35A	(1)	25.10.61	N.S.	++	++	1/20	Neg.	1/80	Pos.
" "	(2)	" " "	N.S.	++	++	1/20	Neg.	1/80	Pos.
"	(3)	" " "	N.S.	++	++	1/20	Neg.	1/80	Pos.
"	(4)	" " "	++	++	++	1/20	Neg.	1/80	Pos.
38A	(1)	3.11.61	++++	++++	++++	1/320	1/80	1/1280	Pos.
"	(2)	" " "	++++	++++	++++	1/320	1/80	1/1280	Pos.
"	(3)	" " "	++++	++++	++++	1/320	1/80	1/1280	Pos.
"	(4)	" " "	++++	++++	++++	1/320	1/80	1/1280	Pos.
39A	(1)	3.11.61	++++	++++	++++	1/640	Neg.	1/20	Pos.
"	(2)	" " "	++++	++++	++++	1/640	Neg.	1/20	Pos.
"	(3)	" " "	++++	++++	++++	1/320	Neg.	1/20	Pos.
"	(4)	" " "	++++	++++	++++	1/320	Neg.	1/20	Pos.
40A	(1)	3.11.61	++++	++++	++++	1/1280	1/160	1/640	Pos.
"	(2)	" " "	++++	++++	++++	1/1280	1/160	1/640	Pos.
"	(3)	" " "	++++	++++	++++	1/1280	1/160	1/640	Pos.
"	(4)	" " "	++++	++++	++++	1/1280	1/160	1/640	Pos.
41A	(1)	3.11.61	++++	++++	++++	1/160	1/40	1/640	Pos.
"	(2)	" " "	++++	++++	++++	1/160	1/40	1/640	Pos.
"	(3)	" " "	++++	++++	++++	1/160	1/40	1/640	Pos.
"	(4)	" " "	++++	++++	++++	1/160	1/40	1/640	Pos.

M.R.T. = Milk ring test.

M.P.T. = Milk plate test.

M.C.T. = Milk Capillary tube test.

M.W.T. = Milk whey tube agglutination test.

Neg. = Negative.

Pos. = Positive.

N.S. = The Samples were not suitable for the test.

+ = Indicates the nature of positive reaction.

TABLE 5. MILK FROM A HERD ASSOCIATED WITH HUMAN BRUCELLOSIS.

Sample No.	Date Collected	DIRECT INVESTIGATION. SEROLOGICAL EXAMINATION.				BIOLOGICAL EXAMINATION. SEROLOGICAL TESTS.		Culture ex organs.
		M.R.T.	M.P.T.	M.C.T.	M.W.T.	1st.Bleeding	2nd.Bleeding	
3B	1.5.63	++++	++++	++++	1/40	1/20	1/80	Pos.
4B	1.5.63	+++	+++	+++	1/20	Neg.	Neg.	Neg.
6B	1.5.63	++	+	+	Neg.	Neg.	Neg.	Neg.
8B	1.5.63	++++	++++	++++	1/80	1/640	N.T.	Pos.
13B	1.5.63	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
18B	9.5.63	++	+	+	1/4	Neg.	Neg.	Neg.
36B	9.5.63	+++	++++	++++	1/160	Neg.	Neg.	Neg.
44B	9.5.63	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.
45B	9.5.63	++	+++	+++	1/160	1/320	N.T.	Pos.
47B	9.5.63	++	++	++	1/4	Neg.	Neg.	Neg.
50B	9.5.63	++	++++	++++	1/160	1/160	N.T.	Pos.

M.R.T. = Milk ring test.
M.P.T. = Milk plate test.
M.C.T. = Milk capillary tube test.
M.W.T. = Milk whey agglutination test.

Neg. = Negative.
N.T. = Not tested.
Pos. = Positive.
\+ = Indicates the nature of positive reaction.

TABLE 5A. RESULTS OF THE RING TEST BY STANDARD AND BY MODIFIED PROCEDURES.

Sample No.	Date Collected.	MILK RING TEST		
		INCUBATION AT 37°C.	CENTRIFUGATION AT	
			100 r.p.m.	3000 r.p.m.
3B	1. 5.63	++++	++++	++++
4B	1. 5.63	+++	+++	+++
6B	1. 5.63	++	++	++
8B	1. 5.63	++++	++++	++++
13B	1. 5.63	+	+	++
14B	9. 5.63	Neg.	Neg.	++
18B	9. 5.63	++	++	++
26B	9. 5.63	Neg.	Neg.	++
36B	9. 5.63	+++	+++	+++
44B	9. 5.63	Neg.	Neg.	++
45B	9. 5.63	++	++	++
47B	9. 5.63	++	++	++
50B	9. 5.63	++	++	++

M.R.T. = Milk ring test.

M.P.T. = Milk plate test.

M.C.T. = Milk Capillary tube test.

M.W.T. = Milk whey agglutination test.

Neg. = Negative.

Pos. = Positive.

\+ = Indicates the nature of positive reaction.

TABLE 6: CERTIFIED MILK,(GROUP 1).

Nature of reaction.	Number of samples.			
	Ring test.	Plate T.	Capillary T.	Whey T.
a. Strongly positive	9 (9)	6 (6)	5 (6)	-
b. Positive	7 (7)	5 (4)	8 (6)	8 (10)
c. Weakly positive	2 (3)	5 (5)	3 (3)	-
d. Doubtful	5 (4)	5 (5)	5 (4)	-
e. Negative	91 (91)	93 (94)	93 (95)	106 (94)
Total number of samples	114 (114)	114 (114)	114 (114)	114 (114)
Aggregate of positive reaction	23 (23)	21 (20)	21 (19)	8 (10)
Percentage of positive samples	20.18 (20.18)	18.42 (17.54)	18.42 (16.67)	7.02 (8.77)

Figures in brackets embody the results in Table 1A.

TABLE 7: BULK RAW MILK, (GROUP 2).

Nature of reaction.	Number of samples.			
	Ring test.	Plate T.	Capillary T.	Whey T.
a. Strongly positive	7	1	1	-
b. Positive	12	1	2	14
c. Weakly positive	15	10	6	-
d. Doubtful	5	7	12	-
e. Negative	39	59	57	64
Total number of sample	78	78	78	78
Aggregate of positive reactions	39	19	21	14
Percentage of positive samples	50.0	24.4	26.9	17.9

TABLE 8. PASTEURISED MILK, (GROUP 2).

Nature of reaction.	Number of samples.			
	Ring Test.	Plate T.	Capillary T.	Whey T.
a. Strongly positive	-	-	-	-
b. Positive	2	-	-	11
c. Weakly positive	12	10	7	-
d. Doubtful	15	5	7	-
e. Negative	21	35	36	39
Total number of samples	50	50	50	50
Aggregate of positive reactions	29	15	14	11
Percentage of positive samples.	58.0	30.0	28.0	22.0

TABLE 9. SAMPLES FROM INDIVIDUAL ABORTING ANIMALS, (GROUP 3).

Nature of reaction.	Number of samples.			
	Ring Test.	Plate T.	Capillary T.	Whey T.
a. Strongly positive	18	17	19	-
b. Positive	3	2	2	22
c. Weakly positive	1	4	3	-
d. Doubtful	2	-	3	-
e. Negative	32	33	29	34
Total number of samples examined	54	56	56	56
Aggregate of positive reactions	24	23	27	22
Percentage of positive samples.	44.44	41.1	48.21	39.3

TABLE 10. INDIVIDUAL SAMPLES OF CERTIFIED MILK, GROUP 4.

Nature of reaction.	Number of samples			
	Ring Test.	Plate T.	Capillary T.	Whey T.
a. Strongly positive	2	4	4	-
b. Positive	2	2	2	8
c. Weakly positive	5	1	1	-
d. Doubtful	1	2	2	-
e. Negative	41	42	42	43
Total number of samples.	51	51	51	51
Aggregate of positive reactions	10	9	9	8
Percentage of positive samples.	19.6	17.7	17.7	15.7

T. = Test.

TABLE 11. BIOLOGICALLY POSITIVE SAMPLES AND THEIR SEROLOGICAL REACTIONS.

NATURE OF REACTION OBSERVED BY	NUMBER OF BIOLOGICALLY POSITIVE MILK SAMPLES.				TOTAL OF BIOLOGICALLY POSITIVE SAMPLES IN FOUR GROUPS.
	1st. group	2nd. group	3rd. group	4th. group	
RING TEST.					
a. Strongly positive	9	3	11	2	25
b. Positive	5	1	1	-	7
c. Weakly positive	1	2	-	2	5
d. Doubtful	2	-	-	-	2
e. Negative	1	2	1	1	5
Total of biologically positive samples.	18	8	13	5	44
PLATE TEST.					
a. Strongly positive	6	1	11	3	21
b. Positive	3	-	1	1	5
c. Weakly positive	4	3	1	-	8
d. Doubtful	2	1	-	-	3
e. Negative	3	3	-	1	7
Total of biologically positive samples.	18	8	13	5	44
CAPILLARY TEST.					
a. Strongly positive	6	1	11	3	21
b. Positive	4	-	1	1	6
c. Weakly positive	3	2	1	-	6
d. Doubtful	3	1	-	-	4
e. Negative	2	4	-	1	7
Total of biologically positive samples.	18	8	13	5	44
WHEY TEST.					
a. Positive	8	3	12	4	27
b. Negative	10	5	1	1	17
Total of biologically positive samples.	18	8	13	5	44
Percentage of biologically positive samples in each group.	(15.78)	(10.26)	(23.21)	(9.8)	-

TABLE 12.

COMPARISON OF THE POSITIVE BIOLOGICAL EXAMINATIONS WITH THOSE OF THE SEROLOGICAL TESTS.

GROUPS	(1) BIOLOGICALLY NEGATIVE BUT SEROLOGICALLY POSITIVE SAMPLES.				(2) BIOLOGICALLY AND SEROLOGICALLY POSITIVE SAMPLES.				(3) BIOLOGICALLY POSITIVE BUT SEROLOGICALLY NEGATIVE SAMPLES.			
	R.T.	P.T.	C.T.	W.T.	R.T.	P.T.	C.T.	W.T.	R.T.	P.T.	C.T.	W.T.
Group 1: Tables (1 & 1A).	14	13	10	4	17	15	16	8	1	3	2	10
Group 2: Table (2)	33	14	17	11	6	5	4	3	2	3	4	5
Group 3: Table (4)	12	10	14	10	12	13	13	12	1[S]	-	-	1
Group 4: Table (5)	6	5	5	4	4	4	4	4	1	1	1	1
Total number of samples.	65	42	46	29	39	37	37	27	5	7	7	17

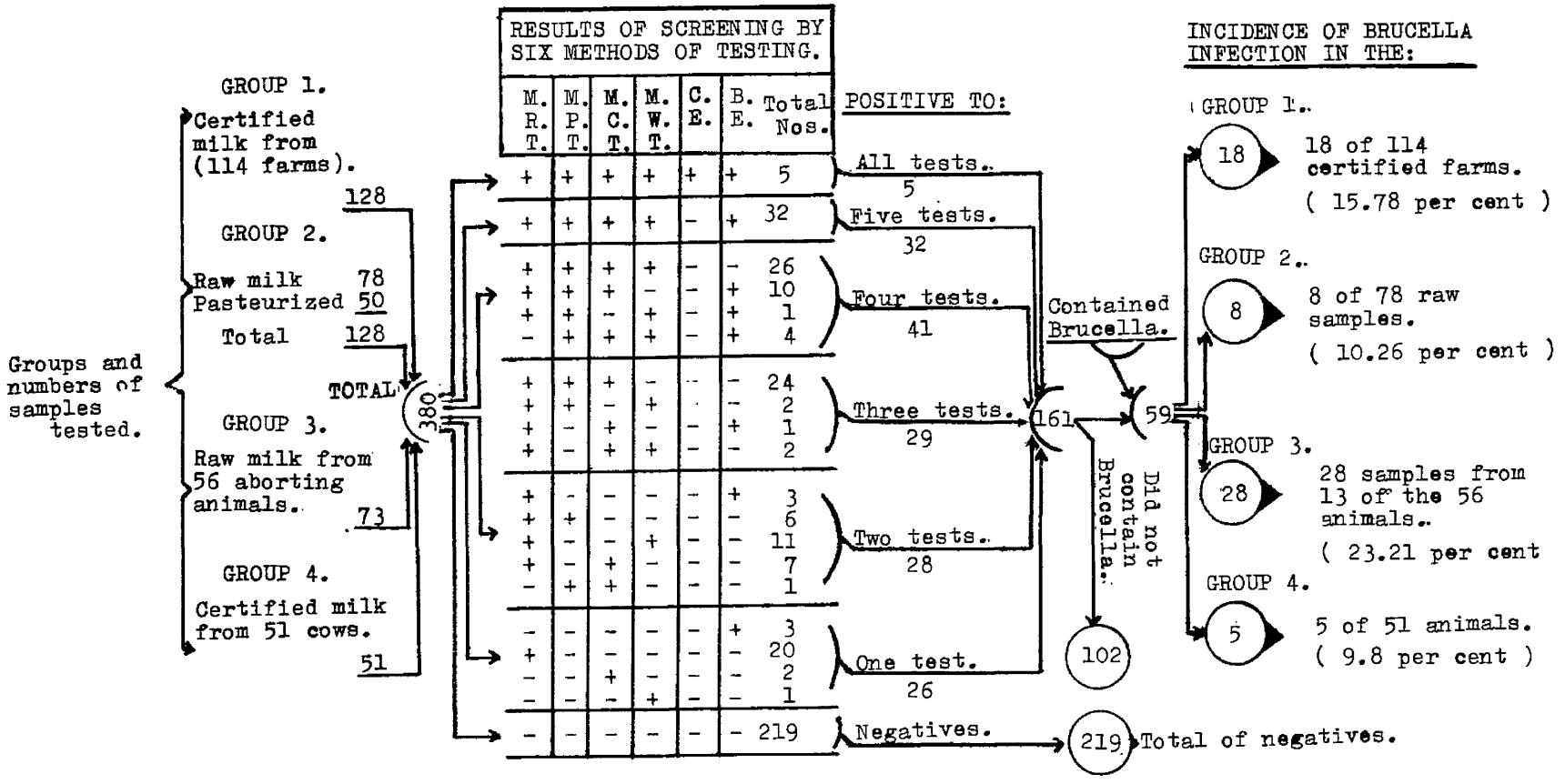
Since none of the pasteurised milk samples in the third group gave positive biological results, they are not included in this table.

S = Milk sample which was watery and not suitable for the ring test.

R.T. = Ring test. C.T. = Capillary tube test.

P.T. = Plate test. W.T. = Whey test.

TABLE 13. A SUMMARY OF ALL THE RESULTS



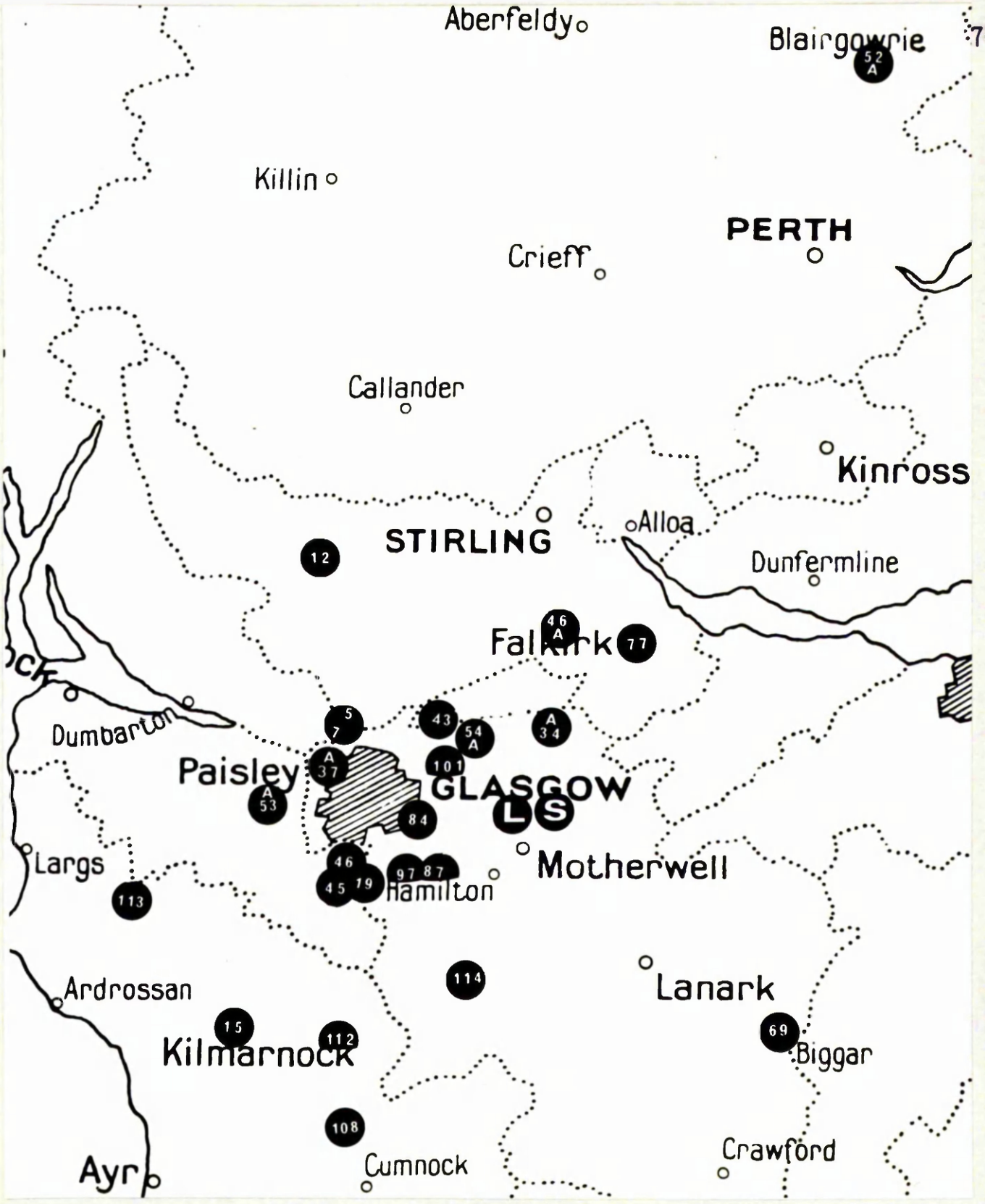


Fig. 12: The location of the farms from which <u>Brucella</u>-infected milk was procured. The numerals indicate the numbers of milk samples obtained from the farms.

L = 38A, 39A, 40A and 41A. S = 3B, 8B, 44B and 50B. Samples Nos. 1A and 4A were obtained from a farm in Nairnshire and Sample No. 35A came from Banffshire.

isolated./

5. DISCUSSION.

A. MICROSCOPICAL EXAMINATION.

With the aid of selective differential staining, Brucellae were not readily demonstrable under the microscope in films made from a mixture of the cream and sediment obtainable after centrifugation. The microscopical mode of examination was discontinued after it had been applied to 114 original samples of certified milk in the first group because, by then, only two specimens (1.77 per cent) had been found to be of possible diagnostic significance. Certainly, Brucella micro-organisms were later recovered from both samples via guinea-pigs but, had a diagnosis been made on microscopical evidence alone, only an inconclusive report would have been justifiable in each instance. In passing, it has to be observed that as a sequel of biological examination Brucellae was obtained from 15 other samples in which the micro-organism was not detected under the microscope.

B. DIRECT CULTURAL EXAMINATION.

On only 5 occasions did cultural examination result in the recovery of Brucella micro-organisms from the samples. One (0.87 per cent) was of mixed certified milk, one (1.78 per cent) consisted of individual milk from aborting animals and three (5.88 per cent) were individual samples of certified milk. The recoveries were made via

via/

serum dextrose agar that contained Polymyxin B. and Actidione (S.D.A.). The medium did not prove satisfactory for the isolation of Brucella micro-organisms from heavily contaminated samples.

When serum dextrose agar without antibiotics (S.D.) was used, most of the plates were overgrown by contaminants, such as Gram-positive cocci, Corynebacteria, Gram-negative bacilli and moulds. In the case of S.D.A. medium, the plates were seldom overgrown by moulds but, the same contaminants could also grow, to a lesser extent, on that medium. The high percentage of cultural recovery of Brucellae from samples of milk in the fourth group suggests that the chance of cultivation from individual certified milk is greater than that from mixed certified milk. Even in the case of individual samples the percentage of cultural recovery was not as high as that which attended the biological method of isolation. According to Morgan (1960) the more plates used for each sample the greater is the chance of recovering the micro-organism. He stated that six plates per milk sample would be a realistic number.

Mair (1955) used a medium, which contained only Polymyxin B. and Actidione in the same concentrations as were used in the present experiment but found that the method was impracticable as a routine procedure because of the large number of contaminants encountered.

encountered./

C. SEROLOGICAL EXAMINATION OF MILK.

(I) MILK RING TEST.

In Table 14, infra, are mustered the ultimate results of the data that are detailed in tables 6-10 (Pages 72 to 74), which results are expressed in percentages of the total number of samples originally examined. On average, the milk ring test emerges ahead of the other

TABLE 14.

GROUPS OF MILK.	PERCENTAGES OF POSITIVE SAMPLES GIVEN BY THE SEROLOGICAL TESTS.			
	Ring Test.	Plate Test.	Capillary	Whey T.
1. Certified.	20.18	17.54	16.67	7.02
2. Bulk raw.	50.0	24.4	26.9	17.9
3. Pasteurised.	58.0	30.0	28.0	22.0
4. From aborted individuals.	44.44	41.1	43.21	39.3
5. Certified from individuals.	19.6	17.7	17.7	15.7
Average	38.44	26.14	27.5	20.38

three procedures employed for the detection of Brucella agglutinins present in milk. That superiority was highest in the case of bulk raw and pasteurised milk but was scarcely distinguishable in the instance of mixed certified milk and of individual samples. In

In/

application to milk obtained from cases of clinical abortion, the milk ring test proved inferior to the capillary test and was only slightly superior to the plate test and a little more so to the whey test.

Positive results obtained by the four serological tests are compared with those of the positive biological examinations (Table 12, Page 76). The first column of that table shows that a positive reaction given by any of the four serological tests does not necessarily indicate the presence of Brucella micro-organism in milk. As is revealed in that column, totals of 65, 42, and 29 positive samples detected by ring, plate, capillary and whey tests, respectively, gave negative biological and cultural results. On the other hand, Table 1A (Page 65) discloses that the micro-organism may be recoverable from serologically positive samples if the examinations be repeated. Column 2 of Table 12 (page 76) indicates that the milk ring test gave a positive reaction in 39 biologically positive samples, whilst the whey test detected 27 and the plate and capillary tests each exposed 37 samples. Column 3 of the same table shows that, including one abnormal sample, the ring test failed to give a positive reaction in the case of 5 biologically positive samples, that the whey test was negative in the instance of 17 biologically positive samples and that the plate and capillary tube tests each behaved similarly in the case of 7 biologically positive samples.

samples./

Table 11 (page 75) suggests that, if strongly positive reactions be given simultaneously by the ring, the plate and the capillary tests, the chance of isolating Brucella micro-organism from milk is much higher than when weaker reactions are experienced. It also indicates that 25 of the strongly positive samples detected by ring test contained live Brucellae, whilst only 21 of the strongly positive samples detected by both the plate and capillary tests yielded Brucellae. Those findings assign to the ring test some superiority over the other modes of examination.

Table 5A (page 71) is a record of the results obtained with the standard ring test and those forthcoming when that test was modified by centrifugation at slow and at high speeds. With the aid of centrifugation at 3,000 r.p.m., three other samples were detected that had not been revealed by the standard procedure or as result of centrifugation at low speed. All three reactions were of weakly positive kind but one of the samples (No. 44B) later proved positive to the biological test. Those observations suggest that the sensitivity of the milk ring test is promoted by centrifugation at high speed. Moreover, such a modification was found both to facilitate the reading of the test and appreciably to reduce the time required for its performance. Such claims are open to condemnation on the grounds that too few samples were involved but, at least, they warrant further investigation.

investigation./

Todd & Runney (1940) demonstrated that the temperature of pastaurisation did not affect the agglutination-titre of milk from reacting cows. In the present investigation, the whey tube agglutination test served to show that most of the serologically positive pasteurised specimens and the corresponding samples of raw milk gave similar titres of agglutination (Tables 2 and 3, pages 66 and 67). On the other hand, in the case of the ring test the positive reactions encountered with pasteurised milk were ordinarily less pronounced than those of the corresponding raw samples, even when test specimens were made to contain more cream. Those observations may be interpreted as follows.

The effect of the size and the disparity of size of fat-globules in relation to the ring test reaction was emphasised by Ogonowski (1955). As a result of exposure to heat during the process of pasteurisation, the fat-globules of milk tend to coalesce (Aikman 1895) or they may lose their surrounding protein membrane and become free oil (Judkin & Keener 1960). In that way the globules may become either too large or too small and so tend to rise to the top of milk at different rates. Again, the surface area of the fat-globules may be lessened. Microscopical study of the fat-globules of 20 samples of pasteurised milk, made by the writer of this thesis, confirmed the above statements and photomicrographs Nos. 13 & 14 demonstrate the

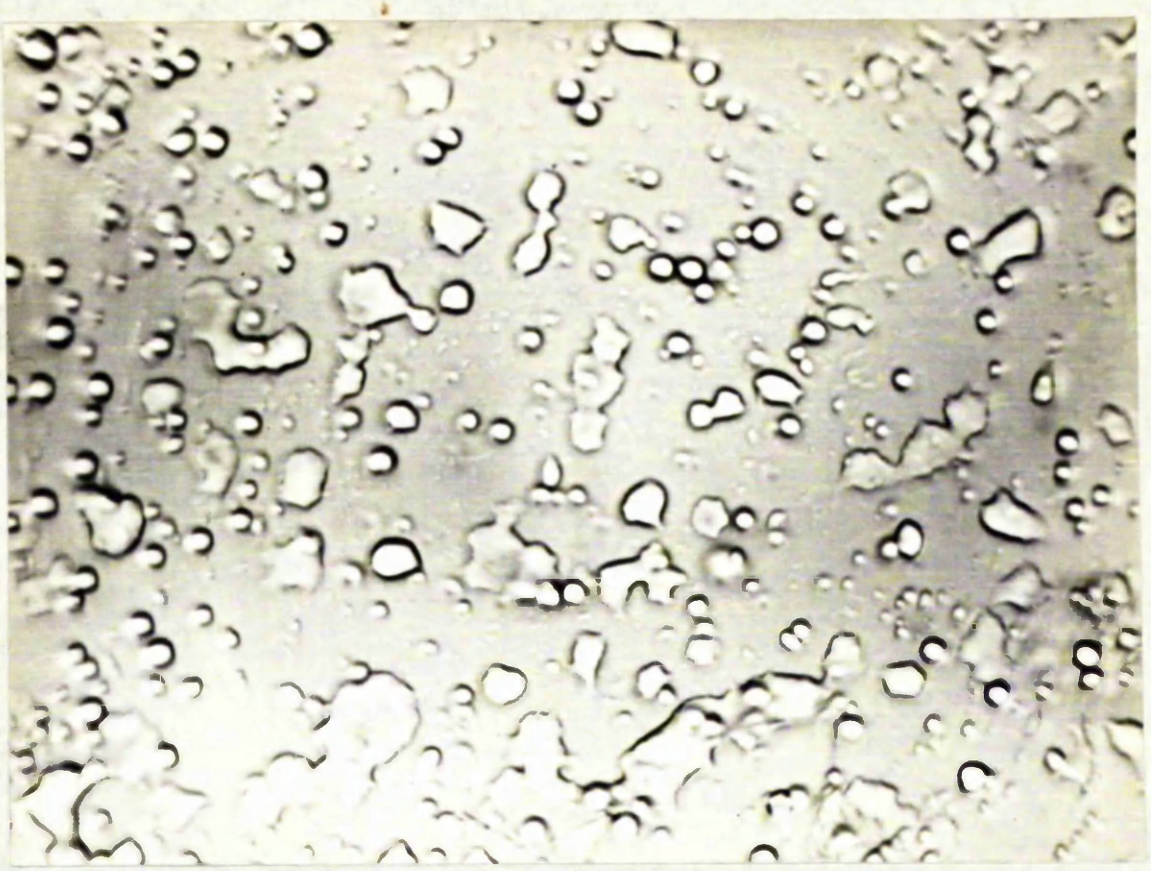


Fig. 13: Large fat-globules of one sample of pasteurized milk (x 600).

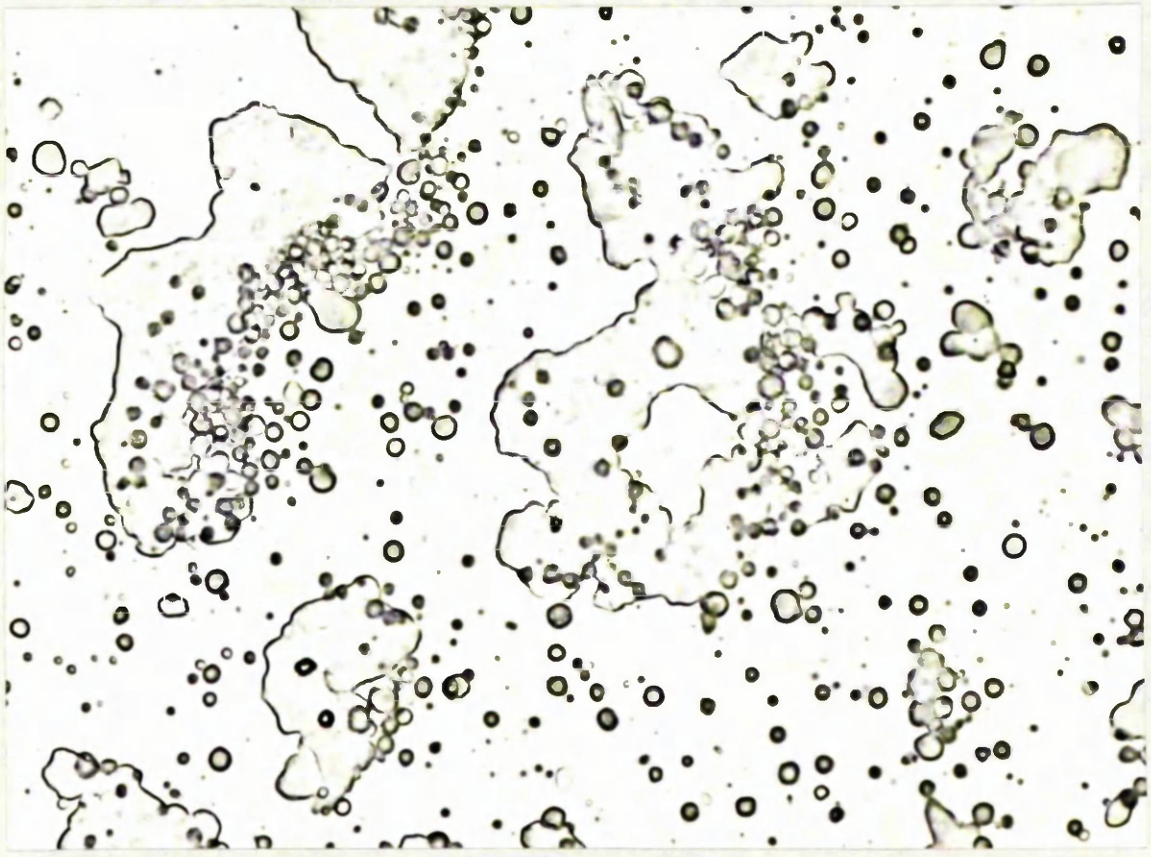


Fig. 14: Large fat-globules of another sample of pasteurized milk (x 600).

the/

large globules that were found in two of the specimens studied. Those two alterations in milk may affect the reaction of the ring test.

The reaction of the ring test is considered to result, mostly, from filtration and, partly, from the attachment of the stained agglutinated antigen to the fat globules. The following evidence is offered in support of that opinion:

(a) It is fairly widely accepted that the ring test is more sensitive when it is applied to mixed milk than it is to milk derived from individual animals. It tends also to give false positive results, especially in the case of milk drawn from an animal at the end, or at the beginning, of a lactation period.

(b) According to Ogonowski (1955), if the size of fat-globules lies between 5 and 7 microns, the test is much more reliable. Judkins & Keener (1960) have stated that the size of butterfat globules in milk varies not only with the breed of cow but also with the stage of lactation. When a cow is going dry before calving, the fat globules become of very small size. Thus, in a mixed sample of milk, the globules are more likely to be of suitable size and so tend to be more uniform in their rate of rising. Consequently in such a milk the process of filtration is likely to be more effective. The higher incidence of positive results in the case of bulk raw milk and of pasteurised milk, (Table 14, Page 81) confirms the value of that test

test/

in application to mixed samples and also indicates that the test enables agglutinins to be detected in milk even when the latter is highly diluted.

The role of fat-globules themselves and the significance of their size in relation to the various results given by the milk ring test may be explained as follows:

(1) When a milk with suitable fat-globules but lacking in agglutinins is subjected to the ring test, the stained micro-organisms in the form of individuals are able to pass through the spaces between contiguous fat globules and so are not carried upwards. Thus, the test gives a negative result.

(2) If such a milk contains homologous agglutinins, the stained Brucellae will clump so that, in addition to a tendency to become attached to the fat-globules, the large clusters will not pass through the spaces between the fat-globules but are trapped and carried upwards. On the other hand, when most of the fat-globules in milk are of large size, the inter-spaces between them are wider so that some, or all, of the agglutinated stained antigen escapes and the test will give a weakly positive or a negative result.

(3) In a milk without agglutinins, when the fat globules are too small, the interspaces between them are so narrow that some of the stained Brucellae, in the form of individuals, may be carried

carried/

up by the fat-globules and the test shows a false position, or a doubtful, result. In such an instance, the tendency of the fat-globules to rise is reduced whereby they take longer to come to the surface. The fat-globules of goat's milk and of homogenised bovine milk ascend less rapidly than do those of normal cow's milk.

(4) The Scherm - Gorlish reaction (1936), which is designed to differentiate between raw and heat-treated milk, also gives the best results with mixed milk. In that test, a suspension of guinea-pig, or of dog, erythrocytes or of bone charcoal is added to a sample under test. With raw milk the corpuscles or the particles of charcoal rise with the cream to give a red or a black ring, respectively. According to Scherm, the test is suitable only for cow's milk and does not apply to goat's milk. Moreover, a visible reaction does not become manifest in under two hours at 37°C. Such a delay may be due to the weight of the red blood cells or of the bone charcoal, both of which are heavier than clumped Brucella micro-organisms and so are carried very slowly to the top by the globules of fat. The relation between the mechanism of that test and that of the milk ring test has been suggested by Van Drimmelen (1951) and other workers.

If the above statements do not fully elucidate the formation peculiar to the milk ring test, they provide a partial explanation of the phenomenon.

phenomenon./

As indicated in Tables 4 and 4A (Pages 68 & 69), the plate and the capillary tests revealed that samples Nos. 16A, 52A and three of the four test samples, No. 35A, were positive. The whey test also showed that the three test samples contained agglutinins and to a titre of 1 in 20 but the ring test was found to be unsuitable in all these instances. Those samples were watery and contained little, if any, butterfat. In the case of samples with unsuitable butterfat, the nature of the reaction is effected so that milk samples which contain little agglutinin may be missed by this test. The inferiority of the ring test to the capillary tube test (Table 14, Page 81), in application to milk obtained from cases of clinical abortion, is a case in point. Also, in the case of samples Nos. 36B, 45B and 50B (Table 5, Page 70), which were procured from individual cows, the reaction of ring test was less pronounced than that given by either of the plate and capillary test. Thus the efficiency of the ring test depends primarily on the quality of a milk sample and, particularly, on the physical state of the butterfat.

(II) PLATE TEST.

A comparison of the results of the plate agglutination test with those of the standard ring test indicates that the former possessed several advantages, namely:

namely:/

(a) It can be used for the detection of Brucella agglutinins in milk irrespective of its content of butterfat or in milk which has been altered in consequence of mastitis or of fermentation or of homogenisation.

(b) It can be conducted at room temperature and also in the field.

Despite the aforementioned advantages, the sensitivity of the milk plate test is diminished by reason of its inability to disclose samples of milk with a low content of agglutinin. Thus, 41 samples (Tables 1 - 5) were found to be negative by means of the plate test but all responded in some degree to the milk ring test. Personal judgement plays an important role in reading the results of the plate test.

(III) CAPILLARY TUBE TEST.

As compared with the ring test, the following advantages attach to the capillary tube test.

(a) Like the milk plate test, the capillary tube test is applicable under field conditions and to milk of almost any physical state of quality. In addition, it involves the use of inexpensive apparatus together with minimal quantities of milk and of antigen and a result is forthcoming in half the time necessary with the ring test.

The capillary tube test has the same disadvantages as is assessed by the plate test, and was found to be negative in 39 samples

samples/

(Tables 1 - 5, Pages 64 to 70), which were either positive or doubtful to the ring test.

The results of the present experiment suggest that the capillary tube test is less sensitive than the milk ring test but is more reliable than the plate test. Hence in any scheme of eradication of brucellosis it would be advisable to use the ring test together with the capillary tube test for the purpose of screening milk samples.

(IV) WHEY TUBE AGGLUTINATION TEST.

The average percentage of positive results obtained by this test throughout the investigation was 20.38 (Table 14, Page 81), a figure that is inferior to that obtained with any of the three other serological methods employed.

Column 3 of Table 12 (Page 76) also attests the relative inferiority of the whey agglutination test for the disclosure of Brucella-infected animals inasmuch as 17 samples, that were negative to that procedure, proved biologically to contain Br. abortus. Such a failure is more than three times higher than that of the ring test and twice as high as that of the plate and capillary tube tests. Finally, column 2 of Table 12 shows that, relative to the plate, the capillary tube and the ring tests, the ratio of samples which proved positive to both the biological and the whey tests were 0.73 in the case of the plate and the capillary tube tests and 0.69 in the instance of the ring test.

test./

The above evidence suggest that the whey tube agglutination test is not a reliable means for the detection of milk derived from infected animals or herds. On the other hand, 40 of the samples (Tables 1 - 5 Pages 64 to 70) contained Brucella agglutinins although neither biological nor direct cultural examinations revealed the presence of Brucellae. Although most of those samples gave a titre of agglutinins that was less than 1 in 10, there were thirteen in which the titre ranged from 1 in 10 to 1 in 160. Those findings suggest that the detection of Brucella agglutinins by means of the whey test, even to a titre as high as 1 in 160, does not necessarily indicate the presence of Brucellae in milk. That experience bears out the work of Delozet & Menager (1934) who declared that the occurrence of agglutinins in bovine milk is not a criterion that Br. abortus is being eliminated in that secretion. Mention (1940a) also pointed out that the presence of specific agglutinins for Brucella is not an infallible indication of infection of milk. The results of the samples of certified milk (Tables 1 and 1A, Page 64 and 65) confirmed the statement made by Fitch & Bishop (1937) in which they said "If agglutinins for Brucella can be demonstrated in a 1 in 25 dilution in a test of raw market milk, living Brucella can usually be found". Whey agglutination tests, that began with a dilution of 1 in 2, were found to give a wider range suitable for the determination of milk samples that had a very low content of agglutinin. Those samples

samples/

might well have been missed had the test been carried out from a dilution of 1 in 10 upwards. In that way it was possible to detect agglutinins in 29 more samples in the four different groups. Since two of those samples belonging to the second group (Table 2, Page 66) were found to contain Brucellae, the inclusion of lower dilutions appears to be especially valuable in the case of bulk milk in which the individual samples become highly diluted. Again, the results of the last group (Table 5, Page 70) indicate that minimal titres may be encountered in the case of individual milk samples drawn from recently infected animals.

D. BIOLOGICAL EXAMINATIONS.

Table 11 (Page 75) shows that 18 (15.78 per cent) of the certified milk samples in the first group, 8 (10.26 per cent) of the raw bulk samples in the second group, 13 samples (23.21 per cent) from aborting animals in the third group and 5 (9.8 per cent) individual certified milk samples in the fourth group contained Brucellae. Those micro-organisms were isolated from the spleen as well as sometimes from the enlarged inguinal or axillary lymphatic nodes and on two occasions from the liver. Two of the experimental guinea-pigs aborted as a result of infection by Brucella and the micro-organism was isolated from the foetal membranes as well as from the spleen, post mortem. The infected guinea pigs inoculated with milk samples Nos. 12,

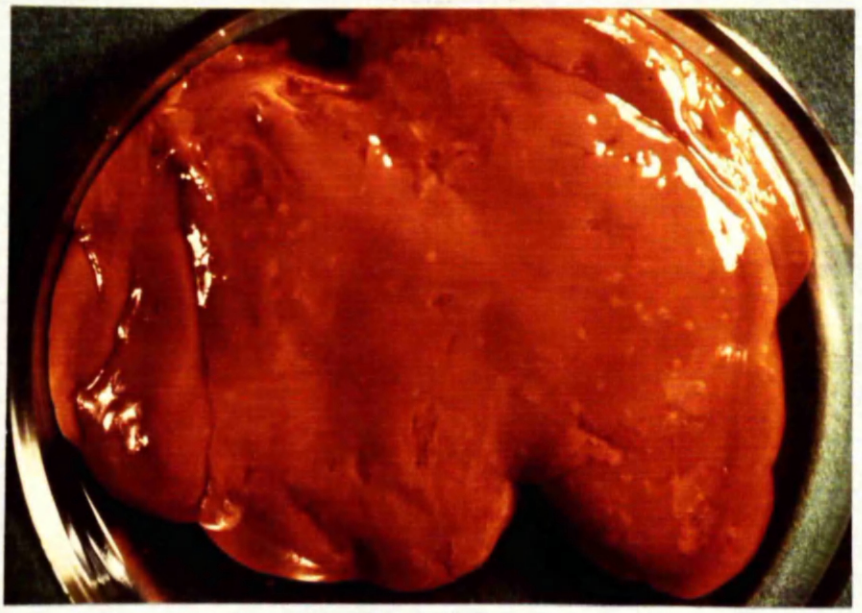


Fig. 15:

Fig. 16:

Figs. above: Infected livers of guinea-pigs Nos. 38R and 39R showing small white spots just below the peritoneum.

12,/

19, 45, 46, 108, 112 and 113 (Table 1 & 1A Page 64 and 65), 38R and 39R (Table 2, Page 66), 29A, 30A, 40A, 41A and 54A (Table 4, Page 68) and 45B (Table 5, Page 70) had greatly enlarged spleens, the size being about two to three times that of the normal animal. Those guinea-pigs also had larger inguinal and axillary lymph nodes. The livers of guinea-pigs Nos. 38R and 39R (Table 2, Page 66) were also larger than normal, and manifested many small white foci that were situated just below the peritoneum (Figs. 15 and 16).

Histopathological study of the livers showed a focal reaction with lymphocytes, some polymorphunuclear leucocytes, occasional giant-cells and some necrosis. There was also a moderate macrophage reaction and occasional giant-cells were found in the sinusoids. Such lesions at the surface of the liver of Brucella-infected guinea-pigs have been reported by Bergfeld (1941). The size of those organs in the other infected guinea-pigs was either normal or slightly larger than normal. Photograph No. 9 shows the size of the large infected spleen and inguinal lymph node compared with those from a normal guinea-pig. Although the sera of guinea-pigs Nos. 69, 101, 5R and 44B proved not to contain any agglutinins, Brucellae were isolated from the spleen of each animal. Guinea-pigs Nos. 101 and 5R were kept for 7 weeks, No. 44B for 8 weeks and No. 69 died after the first bleeding. On the other hand, the s ere of guinea-pigs Nos. 114 and 10R contained agglutinins

agglutinins/

to titres up to 1 in 20 but cultural examinations failed to reveal the presence of Brucella micro-organisms. Since Brucella were later recovered via guinea-pigs from the splenic substances of one of those animals (No. 10R), out of 18 serologically positive guinea-pigs inoculated with 18 samples of milk from the first group, 17 proved biologically positive.

Smith (1932b), in an experiment with 123 guinea-pigs injected with Brucella-infected milk samples, found some guinea-pigs to give positive cultural results and negative serum agglutination tests and vice versa. Ferguson and Robertson (1954) also recovered Brucellae on three occasions from three guinea-pigs, the sera of which proved to be negative to the agglutination test. Menton (1940a) found that, if guines-pig serum was examined for the presence of Brucella agglutinins at three, six and nine weeks after the inoculation of milk, more accurate results were obtained, than if the serum was surveyed only at six weeks after inoculation. Those findings indicate that for biological isolation of Brucella from milk, inoculated guinea-pigs should be kept for a period of 8 - 9 weeks. A period of six weeks, which is mentioned in some literature, is not long enough because in some instances the appearance of agglutinins in serum occurs later. Mair (1955) injected viable Brucella melitensis into guinea-pigs and reported that, although serum titres of two of the guinea-pigs were

were/

1 in 10 and 1 in 1280 resulted, no growth was observed on cultural examination of the spleen. In the present investigation, difficulty was not encountered in the isolation of the micro-organism from the spleen of those guinea-pigs which had a serum titre for Brucella of 1 in 40 or over. In some cases, notably those associated with guinea-pigs that had given negative serological results, two or three colonies were visible after ten days of incubation. Thus a period of 15 days incubation at 37°C. in an atmosphere of carbon dioxide seems to be necessary for the plates which are inoculated with the guinea-pig organs.

Apart from pasteurised milk, there were 60 samples which were considered positive according to one, or more, of the serological tests that were employed but biological examination did not result in the recovery of Brucella micro-organisms. Such an experience may be attributed either to the complete absence of Brucellae from the milk samples or to the presence of an insufficient number of micro-organisms to break down the resistance of the inoculated guinea-pigs. The former view appears to apply especially to the samples of pasteurised milk because none yielded Brucellae whereas 8 of the corresponding raw samples were found to contain the micro-organism. Hence, pasteurisation under standard conditions seems to rid milk completely of living Brucellae. It may be that longer than 7 or 8 weeks is

is/

required for a few Brucella micro-organisms to break down the normal guinea-pig resistance. According to Hutchings & Huddleson (1943), 10 Brucella micro-organisms are sufficient to set up infection in most injected guinea-pigs.

The absence of demonstrable Brucella in serologically positive samples of raw milk suggests either that the animals were not excreting the bacilli by that route or that very few micro-organisms were eliminated. In the latter case dilution with non-infected milk further reduced the number of micro-organism in inocula.

In a small experiment on milk containing agglutinins, 0.01 ml. of the ring test antigen was added to 2 ml. of the sample in a test-tube and the mixture was spun at 3000 r.p.m. for 20 minutes. The intention was to study the distribution of stained antigen in the milk after centrifugation. If live Brucella micro-organism in such a milk behaved like the stained antigen it might indicate which component of the milk would be the best to choose for the inoculum. It was noted that, when the milk sample contained agglutinins, the stained antigen was carried to the top of the milk by the cream but, when the sample did not contain agglutinins, the stained antigen settled on the bottom of the tube. That experiment led the writer to modify the technique of the milk ring test by means of centrifugation.

A total of 207 guinea-pigs was used throughout this experiment. Of those, 11 died after the first bleeding but only three of the 11

11/

yielded Brucella micro-organisms from their spleens. The remaining 8 negative guinea-pigs had been injected with milk samples which gave negative serological test results.

Here it may be mentioned that one tuberculous milk sample was revealed during the survey. The milk sample No. 1R (Table 2, Page 66) was inoculated into one guinea-pig, and 8 weeks after the inoculation, the results of biological examinations were negative for Brucella. Postmortem examination showed some whitish focii just below the surface of the liver and kidney. Microscopical examinations of film of those lesions, stained by the Ziehl-Neelsen Method, revealed the presence of acid-fast bacilli. Further investigation made in the Pathology Department proved that the bovine strain of tubercle bacillus was concerned.

6. SUMMARY.

A total of 380 bovine milk samples was obtained from four different sources, namely:

(a) 128 mixed samples from 114 farms supplying certified milk to the City of Glasgow and its environs;

(b) 128 mixed samples from 27 pasteurising plants in the City of Glasgow. Seventy-eight of those were bulk raw milk and the remaining 50 were pasteurised milk;

(c) 73 samples of milk from 56 individual aborting animals on

on/

various farms in different parts of the West of Scotland and

(d) 51 samples of certified milk from as many cows on one farm associated with a case of human brucellosis.

The above specimens were subjected to the milk ring test, the milk plate agglutination test, the milk capillary tube test, the milk whey tube agglutination test as well as biological, cultural and microscopal examinations. For biological examination, one or occasionally two guinea-pigs were used for each serologically positive sample. In the case of serologically negative samples, one guinea-pig was employed for a pooled mixture of two or three samples. Occasionally, one guinea-pig was inoculated with a particular serologically negative sample. Almost all the experimental animals were 600 grammes in weight, or over. The results were as follows:

(1) Of the 114 original samples concerned in the first group:

23 (20.18 per cent) were positive to the milk ring test,
20 (17.54 do.) do. plate test,
19 (16.67 do.) do. capillary tube test,
10 (8.77 do.) do. whey tube test,
18 (15.78 do.) do. biological test and
1 (0.87 do.) do. cultural examination;

(2) Of 87 bulk raw samples in the second group:

39 (50.0 per cent) were positive to the milk ring test,

test/

19 (24.4 per cent) were positive to the plate test,

21 (26.9 do.) do. capillary tube test,

14 (17.9 do.) do. whey tube test,

8 (10.26 do.) do. biological test.

of 50 pasteurised samples in that group:

29 (58.0 per cent) were positive to the milk ring test,

15 (30.0 do.) do. plate test,

14 (28.0 do.) do. capillary tube test,

11 (22.0 do.) do. whey tube test and

none to the biological and cultural examination.

(3) Of 56 samples of milk from individual aborting cows in the third group:

24 (44.44 per cent) were positive to the milk ring test,

23 (41.1 do.) do. plate test,

27 (48.1 do.) do. capillary tube test,

22 (39.3 do.) do. whey tube test,

13 (23.21 do.) do. biological test and

1 (1.78 do.) do. cultural examination.

(4) Of 51 samples of certified milk from individual cows in the fourth group:

10 (19.6 per cent) were positive to the milk ring test,

9 (17.7 do.) do. plate test,

test,/

9 (17.7 per cent) were positive to the capillary tube test,

8 (15.7 do.) do. whey tube test,

5 (9.8 do.) do. biological test and

3 (5.88 do.) do. cultural examination.

(5) The milk ring test was found to be appreciably superior to the plate, the capillary and the whey tests and the whey test was inferior to any of the other three serological methods employed. The worth of the milk ring test, modified by means of centrifugation, appeared to exceed that of the standard incubation method.

(6) It was noted that pasteurisation altered the size of the fat-globules of milk and consequently affected the outcome of the ring test. The mechanism of that test was discussed and the reaction was considered to result mostly from filtration and partly from the attachment of the stained agglutinated antigen to the fat-globules of milk.

(7) Some advantages were possessed by both the milk plate agglutination and the capillary tube tests and the latter was found to be the better of the two for detecting the presence of Brucella agglutinins in milk.

(8) The relation between the presence of Brucella agglutinins and that of Brucella micro-organisms in milk was examined with the conclusion that a titre of agglutinins in milk even as high as 1 in 160

160/

did not definitely indicate the occurrence of Brucella. The determination of small amounts of agglutinins in milk, feasible when the whey tube agglutination test was performed with dilutions from 1 in 2 upwards, was found to be especially useful in the case of highly diluted samples.

(9) The methods employed for the direct isolation of Brucella were less satisfactory in application to mixed milk than they were to individual samples and proved distinctly inferior to the biological mode of recovery.

(10) As a means of detecting Brucella in milk, the microscopical examination was disappointing.

PART II. THE TYPING OF RECOVERED BRUCELLAR STRAINS TOGETHER WITH A STUDY OF THEIR VIABILITY AND OF THEIR DISSOCIATION IN ARTIFICIAL CULTURE.

1. HISTORICAL:

Until 1929 there was not any reliable method for the identification of the three species of the genus Brucella. Br. abortus and Br. melitensis were serologically indistinguishable. Evans (1923 - 1925) regarded Br. melitensis and Br. abortus as serological varieties of one species. Huddleson and Abel (1927-1928b) and Huddleson (1931) were the first to report that differentiation of the three established species of the genus could be effected through the medium of:

of/

(1) their ability to produce H_2S and

(2) their sensitivity to bacteriostatic dyes, such as thionin, basic fuchsin, methyl violet and pyronin.

The fact that Br. abortus could produce H_2S from protein, or from amino acids containing sulphur, had already been recognised by Zwick and Zeller (1913).

Although serological procedures had been satisfactorily applied to the classification of many genera of micro-organisms, they had failed with the Brucella group until a solution of the problem was offered by Wilson & Miles (1932). Those workers proved that it was possible to differentiate Br. abortus from Br. melitensis by means of absorption of agglutinins, provided that absolutely smooth strains of Br. abortus or of Br. melitensis were employed for the production of homologous antisera. Wilson (1933) studied the serological behaviour of 155 strains of Brucella cultures collected from different parts of the world and classified them into the following groups:

(a) Bovine abortus with 5 sub-groups;

(b) Porcine abortus with 2 sub-groups;

(c) Melitensis with 2 sub-groups;

(d) Para-abortus with 2 sub-groups and

(e) Para-melitensis.

Para-melitensis/

The two latter groups were considered to be rough variants of the corresponding species. Wilson finally recommended that for classification, every strain of Brucella should be examined with respect to CO_2 requirement, H_2S production, growth in the presence of bacteriostatic dyes and antigenic structure, which formulary has since come to receive almost universal recognition.

McLeod (1944) found that strain 19 of Br. abortus would not grow on media containing thionin-blue in concentrations favourable to the growth of other strains of that species. Cruickshank (1954) confirmed that observation and also pointed out that strains of Br. abortus, which were sensitive to the four usual test dyes, did not grow on media containing thionin-blue. The latter observation was corroborated by Morgan (1961). Since strains of that kind were first described by Wilson (1933), they have come to be designated Type 2, Wilson (Huddleson, 1955).

Wohlfeil & Weiland (1937) and Wohlfeil & Wollenberg (1937) reported that members of the genus Brucella hydrolyze urea to yield one molecule of carbon dioxide and two molecules of ammonia. Bauer (1949) described a test based on differences in urease-activity shown by the three species of Brucella (Hoyer, 1950). Hoyer (1950) reported that such a test might be of further aid in the differentiation of Br. abortus from Br. melitensis and Br. suis.

Br. suis./

The utilization of glucose by Brucella was noted by McAlpine & Slanetz (1928). According to Coleman et al. (1930) and McNutt & Purwin (1931) arabinose, glucose, levulose, galactose and xylose were fermented by Brucella and from arabinose a greater amount of acid was always formed. McCullough and Beale (1951) showed that nine carbohydrates were oxidized by a resting cell suspension of Brucella. Piket & Nelson (1955) studied the carbohydrate fermentation of 91 strains of Brucella and stated that it was possible to differentiate three species of Brucella according to their quantitative effect upon glucose, inositol, maltose, mannose, rhamnose and trehalose. Finally Huddleson (1957), on the basis of biochemical behaviour, classified the genus into the following species and types:

(1) Brucella melitensis, Type 1;

(2) Brucella abortus, Types I, II & III and

(3) Brucella suis, Types I, II & III.

The isolation of anti-Brucella phages, previously described by Russian workers, was announced by Parnas et al. (1958 a & b) who stated that phages lysed cultures of Br. abortus but not those of Br. melitensis or of Br. suis. That finding was confirmed by Stinebring & Braun (1959), Van Drimmelen (1959) and Morgan et al. (1960). After a study of the effect on 329 cultures of Brucella of four phages of Russian origin and of other 34 which he had isolated in Poland, Parnas (1961) declared that there was not any absolute specificity of phages

phages/

for the three species of the genus Brucella and that the phage test was only supplementary to differentiation by biochemical and serological methods.

Meyer & Cameron (1956) compared with Br. melitensis the infective agent of the genital disease of sheep that had been isolated by Simmons & Hall (1953) and by Buddle & Boyes (1953). The comparison was made on a basis of serological and biochemical characteristics together with the metabolic patterns that were forthcoming from Warburg manometric techniques and led to the conclusion that, since the Brucella group possessed homogenous characteristics which differed from those of the ovine organism, the latter should not be placed in the genus, Brucella. The same authors (1961 a,b) showed that each of the three species of Brucella displayed a definitive oxidative metabolic pattern and that the biotypes within each species also displayed a metabolic pattern that was singular for the species, irrespective of any differences in susceptibility to the bacteriostatic action of basic fuchsin and thionin that might obtain. Meyer (1961a) showed that typical strains of Brucella, indistinguishable by conventional biochemical and serological methods, were differentiable by their oxidative metabolism. Furthermore, Meyer (1961 b) and Meyer & Morgan (1962) demonstrated that only those strains that showed the oxidative metabolic pattern of Br. abortus were susceptible to Brucella bacteriophage, Type abortus, Strain 3,

Strain 3/

irrespective of their biochemical and serological behaviour. The authors consequently stated that serological and biochemical methods did not afford entirely reliable means for the identification of Brucella species. In the case of Br. neotoma, Stableforth & Jones (1963) held that the micro-organism fell into the genus Brucella but that its specific status could not be determined as long as only seven cultures were all that had been isolated.

Morgan (1963) examined 3919 Brucella cultures obtained from different parts of the world, including Great Britain, for lysis by five different Brucella phages. By use of a routine test dilution (R.T.D.) and of another quantum equal to 1,000 X R.T.D., he was able to differentiate Br. abortus from Br. melitensis and from Br. suis. Thus, all strains of Br. abortus were found to be lysed by both dilutions of the five phages whereas cultures of Br. suis proved susceptible only to the dilution of 1000 X R.T.D. None of Br. melitensis was lysed by either dilution. The results of phage-typing were found to be identical with the oxidative metabolic patterns characteristic of each of the three species that are obtainable by the Warburg mano-metric techniques. The five Brucella phages concerned were designated Tbilisi ("Tb"), 10/I, 24/II, 212/XV and 371/XXIX. Morgan suggested that the bacteriophagia of Br. suis may be due to the phenomenon of "lysis from without" inasmuch as he found it impossible to propagate phage when that species of micro-organism was used as a

a/

host. Finally, the Sub-committee on Taxonomy of the genus Brucella, reported by Stableforth and Jones (1963), made use of biochemical and metabolic characters, serological behaviour and phage sensitivity to classify the genus into three species and 15 biotypes, as follows:

(a) Brucella melitensis, Types 1, 2 and 3;

(b) Brucella abortus, Types 1, 2, 3, 4, 5, 6, 7, 8 and 9 and

(c) Brucella suis, Types 1, 2 and 3.

2. a MATERIALS:

In all, 48 cultures of Brucella were studied. 43 were isolated by the writer as described in the first part of the thesis whilst the remaining 5 cultures were obtained from foetal material that became available from time to time in the Department and were symbolized by the letters 'c', 'd', 'e', 'f', 'g'. Br. abortus and Br. melitensis mono-specific sera together with a strain of each of the Russian Brucella phage, "Tb", and the Polish phagees, 212/XV and 317/XXIX, and standard strains of Br. abortus, Nos. 544, 19, bovis No.12, as well as of Br. melitensis, No. 16M, were obtained from the Central Veterinary Laboratory, New Haw, Weybridge, Surrey. The concentration of particles per millitre was 10^9 in the case of the Russian phage and 10^6 for the Polish ones. One strain of Br. suis, No. 5061, was obtained from the National Collection of Type Cultures, Central Public Health Laboratory Colindale, London, N.W.9. The media used throughout the experiment were solid serum dextrose agar (

agar/

("S.D." medium) as described by Morgan (1960), and broth containing 5 per cent horse serum and 1 per cent dextrose.

3. METHODS:

All 48 cultures were examined for:

(A) Serological behaviour with mono-specific abortus and melitensis sera;

(B) Biochemical characters, comprising:

(1) CO_2 sensitivity, (2) Production of H_2S, (3) Growth in the presence of bacteriostatic dyes, such as thionin, basic fuchsin and thionin blue, (4) Urease activity and (5) Fermentation of carbohydrates;

(C) Susceptibility to Brucella bacteriophage;

(D) 34 cultures in broth were further investigated for viability after storage at 6° to 9°C. over a period of 9 months, and in the course of that study the percentages of dissociated cells present in the broth were also recorded.

All the cultures employed in the above experiments were examined for dissociation by means of oblique transmitted light according to the method recommended by Henry (1933) and only smooth colonies were so studied.

(A) SEROLOGICAL BEHAVIOUR WITH MONO-SPECIFIC ABORTUS AND MELITENSIS SERA.

1. The plate (or Rapid) agglutination test: On a thoroughly cleaned glass plate, one loopful of culture was suspended

suspended/

in two loopfuls of normal saline solution and subsequently mixed with two loopfuls of abortus or of melitensis mono-specific serum. The results were read after 2 minutes at room temperature, during which the plate was rocked gently backwards and forwards. Suspensions of Br. abortus clumped only after they had been mixed with abortus mono-specific serum and those of Br. melitensis agglutinated only in association with melitensis mono-specific serum. For this test, the cultures used were grown on serum dextrose agar plates for 3 - 4 days in an atmosphere containing 10 per cent of carbon dioxide gas.

2. The tube (or slow) agglutination test: For the preparation of antigen, two plates of serum dextrose agar were seeded from the organism under test and incubated for 3 to 5 days in an atmosphere containing 10 per cent CO_2. Loopfuls of growth were then suspended in a quantum of 2 ml. of normal saline solution and thereafter diluted until the opacity of the suspension was equivalent to that of tube 4 on Brown's scale (approximating 6,000 million cells per ml.).

Two series of seven narrow test tubes were set up in racks and made first to contain 0.5 ml. of dilutions of monospecific abortus and of melitensis sera, respectively, that ranged from 1 in 5 to 1 in 320. After 0.5 ml. of suspension had been added to each tube, the series of final dilutions extended from 1 in 10 to 1 in 640. Results were recorded after incubation at 37°C. for 24 hours.

hours./

(B) BIOCHEMICAL METHODS.

1. Sensitivity to Carbon Dioxide. The test was carried out on all cultures as soon as they had been identified and again on 43 of them after a lapse of 10 to 20 months. In the latter case, the cultures were not freeze-dried but were subcultivated every two months and stored in the refrigerator at 6° - 9°C.

To ensure that all plates were seeded with the same inoculum, one colony of a culture was first sown into serum dextrose broth and the tube was then incubated for three days at 37°C. From that source, three plates of S.D. medium were inoculated. One plate was incubated in an atmosphere containing about 10 per CO_2, and the remaining two were kept under aerobic conditions for three days when readings were made. If growth was observed on the plate incubated in an atmosphere of added carbon dioxide but not on those incubated aerobically, one of the latter was transferred into a CO_2 atmosphere and subjected to further incubation for 3 to 7 days, ere final results were recorded. That procedure was designed to show that failure of growth during aerobic incubation was due to the lack of CO_2 and not to the absence of viable organisms. If growth were manifest on a plate at the time of reading, further incubation was not pursued.

2. Production of Hydrogen Sulphide. This test was also carried out with each culture as soon as possible after isolation. After the surface of a serum dextrose agar slope had been inoculated

inoculated/

with Brucella culture, a strip of sterile white filter-paper impregnated with 10 per cent lead acetate, was inserted into the mouth of the bottle and secured by means of the screw-cap. During subsequent incubation in an atmosphere containing 10 per cent CO_2 the lead acetate paper was changed daily and the amount of blackening continued to be recorded until it ceased to develop.

3. Sensitivity to bacteriostatic dyes. Three batches of serum dextrose agar were prepared. At a temperature of about 55°C., and just before the plates were poured, basic fuchsin was added to one, thionin to another and thionin-blue to the third, in quantity that gave a concentration of 1/25,000, 1/30,000 and 1/500,000, respectively. The solutions of dyes were made up and sterilized immediately before use and poured plates were dried off at 37°C. Each batch of plates was checked for effect upon the standard strains, Br. melitensis, No. 16M, and Br. abortus, No. 544.

For dye-sensitivity, each culture was inoculated into serum dextrose broth and incubated at 37°C. for 3 days, to provide the necessary inocula. Each dye plate was divided into four sections in order that four cultures might be accommodated. One loopful of broth culture was streaked five times, without recharging the loop, over the allotted quarter of the plate and distinctively marked. Inevitably, the stroke nearest to the centre of the plate received a very small amount of inoculum, (Fig. No. 17).

(Fig. No. 17)./

The plates were then incubated at 37°C. in an atmosphere containing 10 per cent CO_2 for 5 days and the results were read and recorded according to the degree of growth, thus:

(a) + positive; growth on line No. 1.

(b) ++ positive; growth on lines Nos. 1 & 2.

(c) +++ positive; growth on lines Nos. 1, 2 & 3.

(d) ++++ positive; growth on lines Nos. 1, 2, 3 & 4.

(e) +++++ positive; growth on lines Nos. 1, 2, 3, 4 & 5.

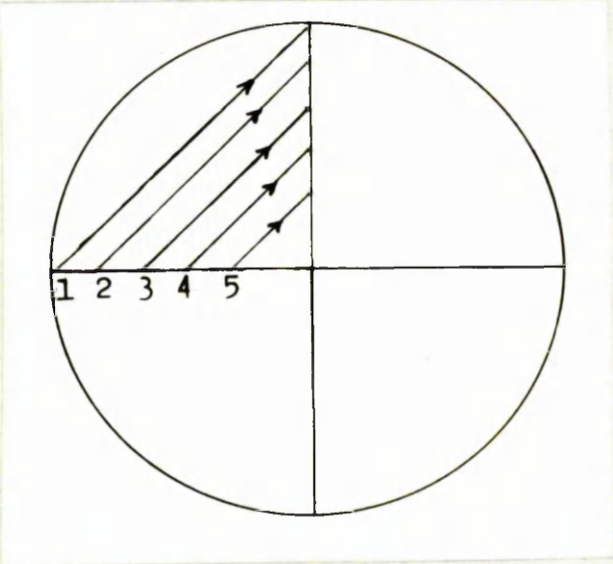


Fig. 17.

The above method is the one used at the Central Veterinary Laboratory, Weybridge. In the case of unsatisfactory results the tests were repeated.

4. Urease activity. For this purpose, use was made of Bauer's medium (Hoyer 1950) and of Christensen's (1946) medium without agar and only 1 ml. of the sterilised solution was used for each

each/.

culture in a bijou bottle. Bauer's medium was employed not only at pH = 4.0, as was originally advised, but also at pH = 7.0. One loopful of the 72 hours' old growth of culture on S.D. medium was inoculated into each of the above media and placed in a water bath at 37°C. for a maximal period of 6 days, ere results were recorded.

5. Fermentation of carbohydrates. This was determined in respect of three sugars, namely: arabinose, xylose and glucose. Assessment was made by means of a solution of 1 per cent of carbohydrate substance in 1 per cent peptone water together with Andrade's indicator. A loopful of culture was inoculated into each of the carbohydrate media, contained in bijou bottles, and incubation was continued at 37°C. for 5 days. Production of acid was indicated by a red colour and any such results were recorded.

(C) PROPAGATION OF BRUCELLA PHAGES AND THEIR USE IN THE TYPING OF BRUCELLA CULTURES.

1. Propagation of Phage. Strains of Br. abortus, No. 544, and Br. bovis, No. 12, provided the cultures appropriate to the production of the Russian phage "Tb" and the two Polish phages, 212/XV and 317/XXIX, respectively. One colony from each strain was inoculated into serum dextrose broth and incubated at 37°C. for 3 days. One plate of well-dried S.D. medium was flooded with the broth culture of strain, No. 544 and two other plates were similarly treated with the broth culture of strain, No. 12, after which all three plates were dried off in the incubator at 37°C. Dilutions of 10^4 phage particles per ml. were

were/

prepared from the above phage specimens. Drops of the [illegible] dilution of each phage were deposited on to the plate seeded with its appropriate propagating culture and allowed to dry. The plates were then incubated at 37°C. for 48 hours in an atmosphere of CO_2. By means of a sterile scalpel blade, areas of confluent lysis from each plate were cut out, transferred to 10 ml. of peptone water and shaken thoroughly. The suspension was then centrifuged at about 3000 r.p.m. and the bacteria were removed by filtration through sintered glass. In accord with the method recommended by Adams (1959), the lowest concentration that produced confluent lysis on solid medium was chosen as the routine test dilution and used for phage-typing of the cultures throughout the experiment.

2. The Typing of Cultures. One colony from each Brucella culture was inoculated into serum dextrose broth and incubated for 3 days at 37°C. The broth cultures were used as inoculum. The plates of S.D. medium were well dried in the incubator and were divided off into four equal sections by marking the base of the plates with a wax-pencil. By means of a sterile capillary pipette, each of the sections was flooded with one of the broth cultures and marked accordingly. To do this, the end of a sterile pipette was bent to form a right angle and about one millilitre of broth culture was drawn into it. The flooding was done by simultaneously expressing the culture from the tip of the pipette and spreading it over the surface of the medium with the

the/

bent end, during which time the plate was held at an angle, with the section to be inoculated downwards, (Fig. 18). Any excess of fluid thus tended to collect on the wall of dish whence it was removed by use of the same pipette. By that procedure, it was possible to flood each section separately without either breaking the surface of the solid medium or transgressing the limits of a section. A fresh pipette was used for each culture. The inoculated plates were placed in the incubator, with the lid partly open, for 1 to 2 hours until their surfaces had completely dried.

Again by means of a wax pencil, each of the four sections was further subdivided into three smaller segments (Fig. 19). With the aid of a Pasteur pipette, one drop (about 0.02 ml.) of each of the three phage suspensions was deposited on each of the segments and the plate was then allowed to dry on the bench. Thereafter, the plates were incubated at 37°C. for 48 hours in an atmosphere containing 10 per cent CO_2 when the results were read. If plates are not properly dried off at the outset, the drops of phage solution tend to remain moist and so spread and coalesce, whereby reading becomes impossible. It may be added that the foregoing procedure enabled an examination to be made of the effect of three, and sometimes of four, different phages on four different cultures (Fig. 19).

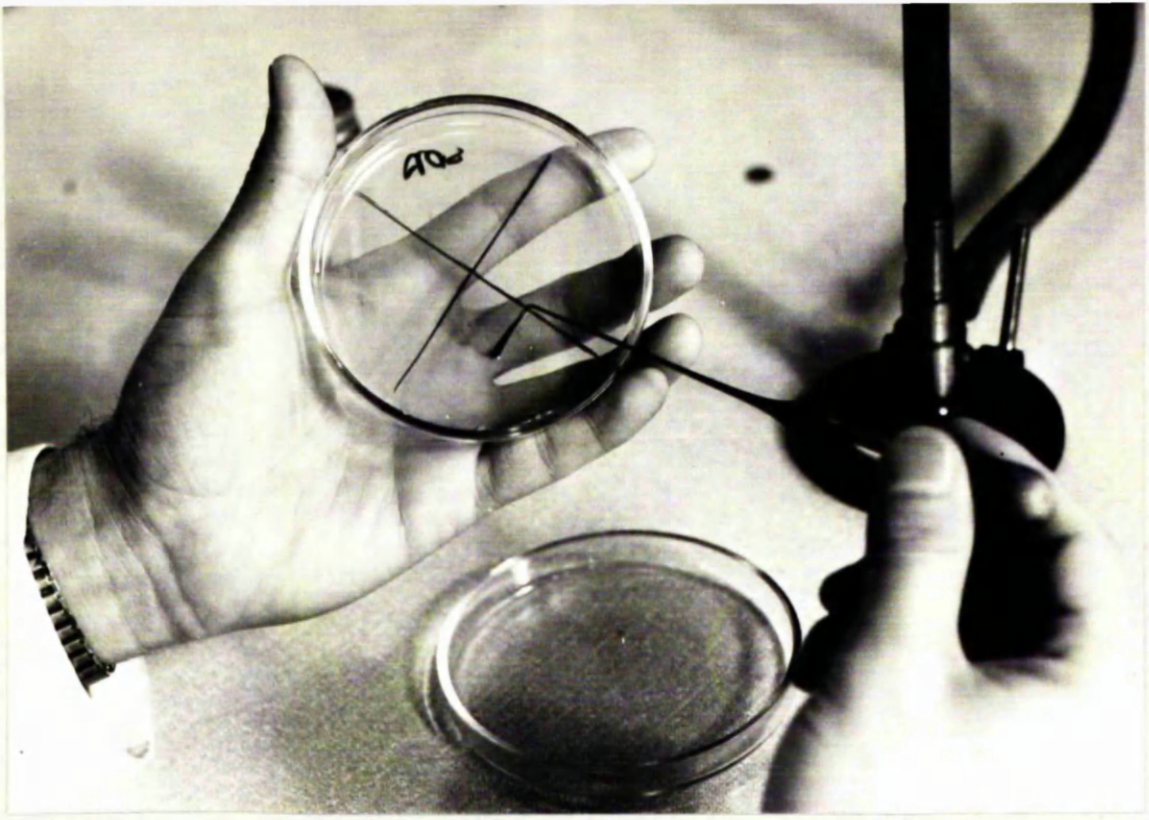
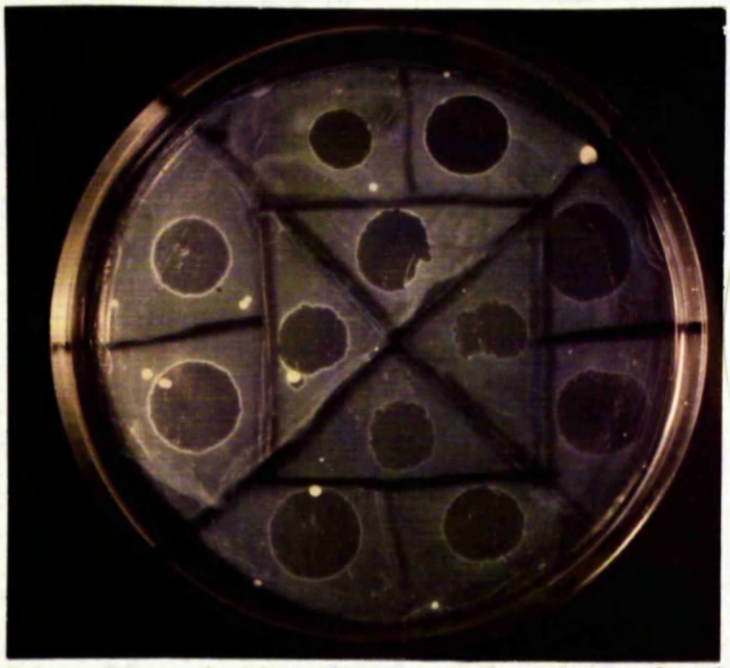


Fig. 18: The method used for flooding a quarter of a plate for phage-typing.



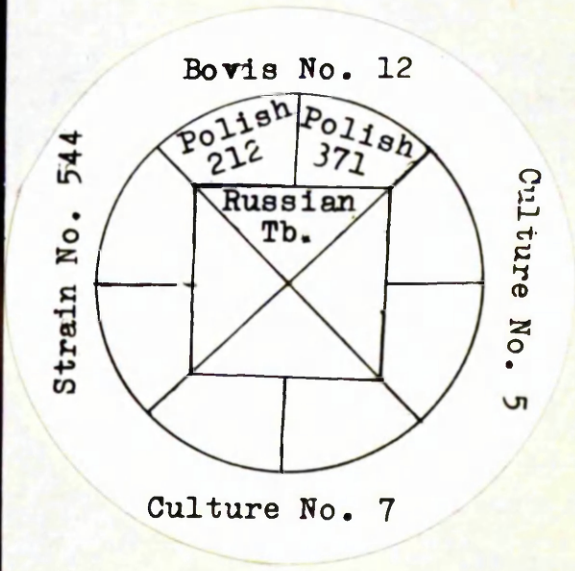


Fig. 19: Lysis observed in phage-typing after incubation at 37°C. for 48 hours in an atmosphere of CO_2. Four cultures were tested by three phages.

(Fig.19)./

(D) VIABILITY AND DISSOCIATION OF BRUCELLA CULTURES IN BROTH.

According to Huddleson (1943), Topley & Wilson (1955) and Stableforth & Galloway (1959), the viability of Br. abortus under various conditions has been studied by diverse investigators, mainly, with particular reference to infective material of either natural or experimental origin. Elberg & Glassman (1947) examined the viability of Br. suis in broth cultures stored at 20 - 25°C. and reported that 25 per cent of the original viable cell count and 100 per cent of virulence were retained for 175 days.

The phenomenon of dissociation in stock cultures of the Brucella group was first noted by Basset-Smith (1921) and was later studied thoroughly by Henry (1923 & 1933), Plastridge & McAlpine (1930), Huddleson (1943 & 1946) and Braun (1946b & 1949). Braun (1946b) found that the addition of the serum or the plasma of normal cows, rabbits, hogs and goats to the broth cultures (in a concentration as low as 2 per cent) prevented the development of dissociated types but that horse and chicken sera were not so effectual. The suppressive effect was found to be due to gamma- and certain beta-globulin fractions of normal bovine plasma. Cole & Braun (1951) reported that sodium pyrophosphate also restrained dissociation and that both Mn^{++} and Mg^{++} increased the rate of the phenomenon. Goodlow et al. (1951) found that D-alanine, too, had an accelerative effect. Huddleson (1956) recorded that dissociation of Br. abortus in liquid media was prevented,

prevented,/

or significantly retarded, if cultures were adequately supplied with air or oxygen.

In the present experiment, 34 of the broth cultures, originally prepared for phage-sensitivity tests, were stored at 6 - 9°C. for the purpose of determining the length of time during which the cultures of Brucella might survive at that temperature. Accordingly, subcultivation to S.D. medium was carried out monthly. From the end of the second month subcultures were also studied for dissociation by counting the number of rough colonies that had developed on the medium. Discrete colonies were obtained by streaking one loopful of the broth cultures on S.D. plates and incubating the latter at 37°C for 5 days in an atmosphere containing 10 per cent CO_2. The number of smooth and rough colonies was then counted and the approximate percentage of each estimated. Colonial study was carried out under the low power objective of a dissecting microscope with the aid of oblique light (Henry 1933) and also by flooding the surface of the medium with crystal violet, in dilution of 1 in 2000. In the latter case, the dye was allowed to act for 15 to 20 seconds ere it was decanted into disinfectant (White & Wilson, 1951). By means of oblique light, smooth colonies appear blue, greenish blue or grey-blue in colour whereas rough colonies are opaque, lustreless and resemble ground glass. After treatment with crystal violet, rough colonies are coloured red or bluish red whilst smooth colonies are pale blue green on a light violet background.

background/

4. RESULTS

Table 15 (p. 123) presents the results of the above tests as applied to 48 Brucella cultures. It indicates that all the cultures but Nos. 43, 10R, 3B and 'f' and 'g', were unable to grow under aerobic conditions after primary isolation. After the lapse of 20, 19, 18, 16, 14, 13 and 6 months respectively, cultures Nos. 87, 3R, 5R, 16R, 39R, 46A and "d" came to grow aerobically but 32 of the cultures retained their sensitivity to carbon dioxide until the end of the period of observation and the remaining four cultures were not tested again.

With the exception of Nos. 43, 3B and 'f' and 'g', all the cultures produced H_2S for a period of from 2 to 6 days and, generally, the amount of the gas was maximal during the second and the third days of incubation. At first, Culture No. 29A was found to form H_2S for more than one month but, when it was subjected to further tests, it produced hydrogen sulphide for not more than 5 days.

Cultures Nos. 43, 3B and 'f' and 'g', were able to grow on medium containing thionin but the remaining 44 strains were sensitive to that dye. Cultures Nos. 12, 87 and C. were susceptible to the action of basic fuchsin and thionin blue to both of which dyes the remaining 45 proved insensitive.

The results of serological examination with abortus and melitensis mono-specific sera, were identical in respect of the rapid and the slow methods. Cultures Nos. 43, 3B and 'f' and 'g', gave a positive agglutinating reaction only with melitensis mono-specific serum. Titres

TABLE 15. RESULTS OF TYPING BY BIOCHEMICAL, SEROLOGICAL AND BACTERIOPHAGIC PROCEDURES.

Culture	CO_2 Requirement		H_2S	Growth on S.D. medium Plus			Agglutination with mono-specific sera.				Fermentation of			Urease Activity						Phage lysis at R.T.D.		
							Abortus		Melitensis					Christensen's		Bauer's medium						
No.	Newly isolated	6-20 months old	Production	Thionin	Basic Fuchsin	Thionin Blue	Slow	Rapid	Slow	Rapid	Arabinose	Xylose	Glucose	medium	day	at pH=4	day	at pH=7	day	Russian Tb.	Polish 212	Polish 317
5	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
7	Pos.	Pos.	6 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
12	Pos.	Pos.	3 days	Neg.	Neg.	Neg.	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	2	Neg.	6	Pos.	6	Pos.	Pos.	Pos.
15	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Pos.	6	Pos.	Pos.	Pos.
19	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
43	Neg.	Neg.	Neg.	+++++	+++++	+++++	Neg.	Neg.	1/80	Pos.	Pos.	Pos.	Neg.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
45	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
46	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
69	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
77	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
84	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
87	Pos.	Neg.	5 days	Neg.	Neg.	Neg.	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Pos.	6	Pos.	Pos.	Pos.
97	Pos.	Pos.	6 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Pos.	6	Pos.	Pos.	Pos.
101	Pos.	Pos.	3 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
108	Pos.	Pos.	4 days	Neg.	++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
112	Pos.	Pos.	3 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
113	Pos.	Pos.	3 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
3R	Pos.	Neg.	2 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
5R	Pos.	Neg.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
10R	Neg.	Neg.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	1	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
15R	Pos.	Pos.	6 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
16R	Pos.	Neg.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
38R	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
39R	Pos.	Neg.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.

Continued on the next page

TABLE 15. RESULTS OF TYPING BY BIOCHEMICAL, SEROLOGICAL AND BACTERIOPHAGIC PROCEDURES.

TABLE 15. (cont'd). RESULTS OF TYPING BY BIOCHEMICAL, SEROLOGICAL AND BACTERIOPHAGIC PROCEDURES.

Culture	CO_2 Requirement		H_2S	Growth on S.D. medium Plus			Agglutination with mono-specific sera.				Fermentation of			Urease Activity						Phage lysis at R.T.D.		
							Abortus		Melitensis					Christensen's		Bauer's medium						
No.	Newly Isolated	6-20 months old	Production	Thionin	Basic Fuchsin	Thionin Blue	Slow	Rapid	Slow	Rapid	Arabinose	Xylose	Glucose	medium	day	at pH=4	day	at pH=7	day	Russian Tb.	Polish 212	Polish 317
45A	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Pos.	6	Pos.	Pos.	Pos.
29A	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
30A	Pos.	Pos.	6 days	Neg.	+++++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
34A	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
35A	Pos.	Pos.	5 days	Neg.	++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
37A	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
38A	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
39A	Pos.	Pos.	4 days	Neg.	++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
40A	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
41A	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
46A	Pos.	Neg.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
52A	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
53A	Pos.	Pos.	4 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
54A	Pos.	Pos.	3 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
3B	Neg.	N.T.	Neg.	+++++	+++++	+++++	Neg.	Neg.	1/80	Pos.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
8B	Pos.	N.T.	5 days	Neg.	++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
44B	Pos.	N.T.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
45B	Pos.	N.T.	5 days	Neg.	++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
52B	Pos.	N.T.	5 days	Neg.	+++++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
c	Pos.	Pos.	5 days	Neg.	Neg.	Neg.	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
d	Pos.	Neg.	5 days	Neg.	+++++	+++++	1/160	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
e	Pos.	Pos.	5 days	Neg.	+++++	+++++	1/320	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
f	Neg.	Neg.	Neg.	+++++	+++++	+++++	Neg.	Neg.	1/80	Pos.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.
G	Neg.	Neg.	Neg.	+++++	+++++	+++++	Neg.	Neg.	1/80	Pos.	Pos.	Pos.	Pos.	Pos.	2	Neg.	6	Neg.	6	Pos.	Pos.	Pos.

TABLE 15. (cont'd). RESULTS OF TYPING BY BIOCHEMICAL, SEROLOGICAL AND BACTERIOPHAGIC PROCEDURES.

Titres/

as high as 1 in 80 were obtained with the slow (or tube) agglutination method. The 44 remaining cultures reacted only with abortus mono-specific serum and to titres that ranged from 1 in 160 to 1 in 320.

Different results in urease-activity were obtained with the cultures on Christensen's and Bauer's media. On the former, all 48 cultures split urea to varying degree but none was so active in Bauer's medium at PH = 4.0 even after incubation in a water-bath at 37°C. for as long as 6 days. In Bauer's medium at PH = 7.0 cultures Nos. 12, 15, 87, 97 and 45R exhibited very weak urease-activity after 2 to 6 days in a water-bath at 37°C.

Save for Nos. 12, 43, 101 and 34A which did not reduce glucose, all the cultures were found to split arabinose, xylose and glucose.

The phage tests on all the cultures gave identical results. At routine test dilution, the Russian "Tb" phage and the two Polish phages 212/XV and 317/XIX, lysed all 48 cultures and formed visible area of lysis on solid media. (Photo 19).

Investigation into the viability and dissociation of Brucella in the 34 broth cultures showed that the number of viable cells began to decline towards the end of the third month and that, after 4 to 4½ months, a count equal to approximately 50 per cent of the original obtained. After 8 to 8½ months, the percentage of viable cells was about 5 and by the end of 10 months thirty of the broth cultures proved sterile after

after/

subculture of two loopfuls to solid medium. Again at the end of 10 months, similar subcultivation from the remaining 4 cultures yielded not more than two colonies. Figure No. 20, infra, consists of the combined viability curves of the thirty-four broth cultures studied.

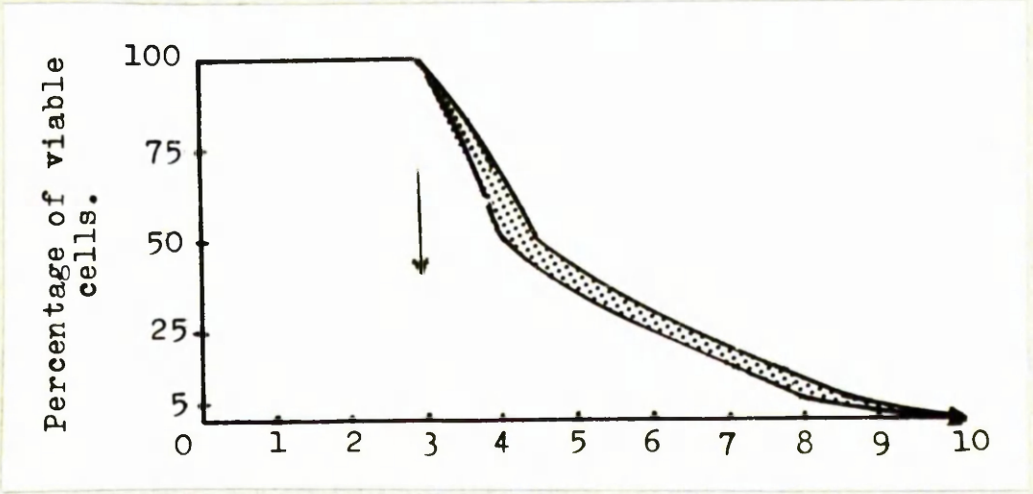


Fig.20. Combined viability curves of 34 broth cultures.

The approximate percentages of rough to smooth colonies encountered on solid media inoculated from the broth cultures were determined monthly from the end of the second month to the close of the eighth month of storage. The results of the first and the last assays, recorded in Table 16, infra, indicate that the rough population

population/

of Brucella cells in the broth cultures tended to increase month by month. Thus, at the end of second month the percentage of dissociated cells in 16 of the broth cultures was 0 - 5; in 12 cultures it was 5 - 10; in 4 it was 10 and in the remaining two cultures, it was 15. At the end of eight months, those percentages had risen, to 5 - 10, 25 - 35, 70 and 75, respectively.

Table 16

Nos. of Broth Cultures	PERCENTAGES OF DISSOCIATED BRUCELLA CELLS IN BROTH CULTURES	
	After Two Months	After Eight Months
16	0 - 5	5 - 10
12	5 - 10	25 - 35
4	10	70
2	15	75

5. DISCUSSION.

Based on conventional methods of differentiation, members of the genus, Brucella, fall into one of the three classical species, namely: Br. abortus, Br. melitensis and Br. suis. Also encountered are some strains which manifest properties belonging to two species and so are not precisely classifiable by the above procedures. The

The/

latter strains may resemble Br. abortus biochemically and be related serologically to Br. melitensis or they may agree biochemically with Br. melitensis and be found to have serological identity with Br. abortus. Some cultures possessed of the biochemical and the serological characters of Br. melitensis may produce H_2S or require added CO_2 for their growth. A few strains have been found to react with both melitensis and abortus mono-specific sera (Stableforth & Galloway, 1959). As a result, the reliability of conventional biochemical and serological methods for the typing of Brucellae has recently been criticized by Meyer & Cameron (1961a & b) and by Meyer (1961a) who introduced the mono-metric method for identification of Brucellar species. Towards the same end, Morgan (1963) developed the method of phage typing for the three classical species.

Throughout the investigation under report, conventional methods of differentiation have been followed in association with typing by phage.

Conventional methods showed that, in serological and biochemical respects, cultures Nos. 43, 38 and 'f' and 'g' were identical with Br. melitensis but all four were susceptible to the three phages at routine test dilution. The remaining 44 cultures were deemed to be Br. abortus because their biochemical characters and their serological behaviour were of that identity. All 44 cultures, except No. 10R, required CO_2 for primary isolation but, after a varying time in

in/

the laboratory, 7 of them became adapted to growth in the absence of that gas. Three cultures appeared to belong to Wilson, Type 2, (Huddleson, 1955) in that they were sensitive to the bacteriostatic action of the three dyes tested. That sensitivity was also confirmed by Morgan (personal communication).

The determination of urease-activity and of sugar fermentation was of little differential value. Thus, distinction between Br. abortus and Br. melitensis was not possible on a basis of urease-activity in Bauer's medium, whether at pH = 4.0 or at pH = 7.0. According to Hoyer (1950), the colour of Bauer's medium at pH = 4.0 is changed by Br. abortus in 2 - 8 hours and by Br. melitensis as well as Br. suis in 15 - 60 minutes. In the investigation under record, all the cultures plus Br. abortus, No.544, Br. melitensis, No. 16M, and Br. suis, No. 5601, gave repeatedly negative results for urease activity in Bauer's medium at PH = 4.0. In that medium at PH = 7.0, 43 of the cultures together with Br. melitensis, No. 16M, and Br. abortus, No. 544, also exhibited complete lack of activity whilst the remaining 5 cultures manifested a feeble reaction. On the other hand, Br. suis, No. 5601, changed the colour of the medium to red within 2 hours. In Christensen's medium in a water-bath at 37°C, Br. suis was effective within 45 minutes whereas a reaction with the cultures under test was delayed for 24 to 48 hours. Br. melitensis, No. 16M,

Table 17. The characters and identity of the 43 strains of Brucella determined by the conventional biochemical and serological methods and also by the three Brucella phages, Russian "Tb.", Polish 212/XV and 317/XXIX.

GROUPS AND DESCRIPTION OF CULTURES.	NOS. OF CULTURES.	RESULTS OF CONVENTIONAL BIOCHEMICAL TESTS.	SUSCEPTIBILITY TO THREE BRUCELLA BACTERIOPHAGES.	SPECIES IDENTITY.
Group 1. Br. abortus type 1, biochemically and serologically.	40	Require CO_2 for isolation; produce H_2S for 2-6 days; grow on basic fuchsin and thionin blue; Did not grow on thionin.	All cultures in 4 groups were lysed.	Brucella abortus.
Group 2. As group 1.	1	Did not require CO_2 for isolation; Produce H_2S for 5 days; Grow on basic fuchsin and thionin blue; Did not grow on thionin.	" " " " " "	"
Group 3. Br. abortus type 2, biochemically and Br. abortus serologically.	3	Require CO_2 for isolation; Produce H_2S for 3-5 days; Did not grow on basic fuchsin, thionin blue and thionin.	" " " " " "	"
Group 4. Br. melitensis type 1, biochemically and serologically	4	Did not require CO_2 for isolation; Did not produce H_2S. Grow on thionin, basic fuchsin and thionin blue	" " " " " "	"

No. 16M/

also gave a positive reaction after 48 hours in the water bath. However, in Bauer's medium at pH = 7.0 and in Christenson's medium, Br. suis, strain No. 5601, with its high urease-activity was readily differentiable from Br. melitensis, No. 16M, Br. abortus, No. 544, and all other cultures under test.

The results of phage-typing suggested that despite the differences in serological and biochemical characteristics all 48 cultures belonged to a single Brucellar species because all were susceptible to the three Brucella bacteriophages at routine test dilution. According to Meyer & Morgan (1962) and Meyer (1962), all 48 cultures should show an oxidative metabolic pattern characteristic of Br. abortus and therefore, should be regarded as that species. In table 17 the 48 cultures have been arranged into four groups according to the results of the various methods used for differentiation. The final identity of the cultures is also determined on the basis of phage typing.

In brief, the results of the foregoing inquiry into the viability of, and dissociation among, thirty-four cultures of Brucella in broth made originally from smooth colonies and stored at 6-9°C. serve to establish that:

(1) After eight to eight and a half months, up to 5 per cent of cells remained viable;

viable/

(2) The proportion of variant cells stood at 5 to 15 per cent at the close of the second month and had increased to 10 to 75 per cent at the end of the eighth month;

(3) The percentage of dissociation was not uniform and was highest in rather less than one-sixth of the cultures (Table 16) and

(4) Even after eight months of storage, smooth colonies of Brucella were recovered.

According to Braun (1946a), Brucella colonies with differing rates of growth exhibit different indices of dissociation and the same author (1947) has stated that dissociation does not occur in the absence of propogation. Topley and Wilson (1955) have described the growth of Brucella at 20°C. as "very slow" so that at 6° - 9°C. reproduction may well be negligible, in which case the variant cells found after two months of storage probably developed while the cultures were initially incubated at 37°C. The increment of variant cells during further storage may be attributable, in part, to the ability of those forms to withstand the lower environmental temperature.

6. SUMMARY.

1. 48 cultures of Brucella were typed by means of conventional biochemical and serological methods and by use of Brucella bacteriophage.

2. Four out of the 48 cultures behaved like Br. melitensis

Br. melitensis/

serologically and biochemically and the remaining 44 resembled Br. abortus serologically. Biochemically, 41 out of 44 belonged to Br. abortus, Type 1, and the remaining 3 to Br. abortus, Type 2. Of 44 strains of Br. abortus, only one grew aerobically on primary isolation and all the remaining 43 needed CO_2 for their development.

3. Neither urease-activity nor fermentative reduction of arabinose, glucose and xylose proved helpful in the typing of cultures.

4. One Russian anti-Brucella phage and two Polish phages were propagated on appropriate bacterial cultures and the routine test dilutions requisite for phage-typing were prepared. The three phages were found to lyse all 48 cultures.

5. Study of cell viability in 34 of the Brucella cultures stored at 6° - 9°C. indicated that, in broth, up to 5 per cent of viable cells survived for as long as eight months.

6. In the case of 34 broth cultures of Brucella originating from smooth colonies and stored at 6° - 9°C., from 10 to 75 per cent of variant cells were demonstrable among the smooth population after a lapse of 8 months.

PART III. THE DEMONSTRATION OF BRUCELLAR BACTERIOPHAGE IN LABORATORY CULTURES.

1. HISTORICAL.

Bacteriophagia was first demonstrated in association with Staphylococci by Twort (1915) and by D'Herelle (1917) in respect of the dysentery bacillus (Shigella shigae). Since then many other pathogenic species of bacteria have been found to be susceptible to transmissible lysis. Gwatkin (1931) appears to have been the first to search for Brucella bacteriophage in material, such as faeces, milk feotuses and blood, which was procured from cows but the results were reported as unsuccessful. Smith (1949) took up the quest anew and directed his efforts towards:

(a) the finding of lysogenic strains among 65 cultures of Brucella;

(b) adaptation of phages active against other species of bacteria so that they might come to lyse Brucella strains and

(c) the discovery of Brucella bacteriophage in 62 samples inclusive of faeces, sewage, uterine fluid from aborted cows and milk. He quite

quite/

failed to demonstrate the presence of phage lytic for Brucella species. Picket & Nelson (1950) reported the observation of distinct plaques in the confluent growth on an agar plate of a mucoid Brucella variant. Moreover several of their stock Brucella cultures, of mucoid type, showed almost complete lysis both in broth and on solid medium after several days of incubation. The phenomenon was ascribed to bacteriophagia even if it was finally reported that the results of further inquiry were not comparable with those typical of the lytic system of coliphage. According to Vershilova (1957), Brucella bacteriophage was first doscovered in Russia by Sorglenko et al. (1939, 1940). Drozhevkina (1957) stated that she had obtained the first stable laboratory strains of Brucella phage in March, 1950. A few years later, Brucella phage was isolated by Drozhevkina and Kiritseva (1955) from both old and freshly isolated strains of Brucella and by Drozhevkina et al. (1957) from the blood of human patients and from aborted foetuses of farm animals. Drozhevkina (1957) has referred to an improved technique for the isolation of Brucella phage and at the same time reported the association of a Vi antigen in Brucella cells with a corresponding Vi phage. She further noted that phage obtainable from laboratory cultures was usually very feeble whereas that recoverable from human patients and from animals was much more active. Mamatsashvili (1957) reported the isolation of 53 strains of Brucella phage from 97

97/

samples of milk, urine and faeces procured from cows that had been vaccinated against brucellosis. He also obtained bacteriophage from anti-brucellosis vaccines. Parnas et al. (1958 a & b) recovered 14 Brucella phages from 200 old Brucella cultures. The phages were described as elementary bodies of globular shape, from 60 to 80 millimicrons in size and without any tail. Morgan et al. (1960) and McDuff et al. (1962) characterised Brucella phage as hexagonal, approximately 65 millimicrons in diameter, with a short wedge-shaped tail, 14 - 16 millimicrons in length.

Parnas (1961) reported the isolation of one Brucella phage from manure and 33 others from old cultures of Brucella. Ostrovskaya (1961) recorded the demonstration of Brucella phage in 126 of 197 Brucella strains. All were feebly lytic and few could be passaged.

The work about to be reported was designed to reveal the presence of Brucella bacteriophage in recovered cultures.

2. MATERIALS AND METHODS.

Of the 41 cultures of Brucella studied, 37 were isolated by the writer in the course of work which has been described in the first part of the thesis. The other four were derived from standard strains of Br. abortus, Nos. S.19 & 544, from Br. melitensis, No. 16M, received from the Central Veterinary Laboratory and from Br. suis, No. 5061, obtained from the Central Public Health Laboratory. The 37 cultures had been maintained in the laboratory for a perio of from 6 to 18 months.

months./

Also investigated were 5 spleens from as many Brucella-infected guinea-pigs, from which source the micro-organism had been isolated ere the organs were stored at 4°C. for 7 to 15 days. Cultures were maintained in serum dextrose agar and serum dextrose broth, as described in the first and the second parts of the thesis, and sintered glass was used for filtration of the liquids.

The methods used were mainly based on the search for lysogenic strains of Brucella i.e. those which carry phage particles with them. The finding of lysogenic strains is the method most generally used for the isolation of bacteriophages from laboratory cultures (Adams 1959). Eight different experiments were carried out:

(1) Following the practice successfully introduced by Fisk (1942) and by Wilson & Atkinson (1945) in respect of Staphylococcal phage as well as by Smith (1949) relative to Brucellar phage, all the cultures were subjected to cross-cultivation. The procedure adopted was as follows.

Each of the 41 Brucella cultures was inoculated into serum dextrose broth and incubated for 48 hours at 37°C. Three plates of well-dried S. D. medium were flooded with one of the broth cultures and dried off in the incubator with the lid of the capsule slightly raised. Each plate was marked off into 16 divisions so that, together, the three plates provided not fewer than 40 sections. One loopful from

from/

the residue of each of the broth cultures was consigned to a segment of medium and its identity marked on the undersurface of the capsule. The same procedure was followed with all of the cultures. After they had been allowed to dry at room temperature, the plates were incubated at 37°C. for 48 hours in an atmosphere containing 10 per cent carbon dioxide, when they were examined for signs of lysis by means of a 16 mm. dry objective as well as by the naked eye.

For the seven other experiments, two kinds of young cultures in serum dextrose broth were employed. Both were grown for only six hours. One culture, hereafter designated "Culture A", consisted of Br. abortus, No. S.19, whilst the other, entitled "Culture B", was composed of a mixture of Br. abortus, Nos. S.19 and 544, Br. melitensis, No. 16M, and Br. suis, No. 5061.

The young cultures were preferred for the reason that, according to Adams (1959), adsorption of phage to host cells proceeds most rapidly when bacteria are in the logarithmic phase of growth.

(2) An experiment was carried out in a manner similar to that used by Parnas et al. (1958 b). From each of the 37 old cultures on serum dextrose agar, a small portion of medium was removed and added to broth Culture A. The 37 mixed cultures obtained in that way were incubated at 37°C. for 48 hours aerobically and thereafter stored at 6°C. for 24 hours. From each mixed culture a few drops were transferred to the original broth Cultures A. and the procedure repeated ten times.

times./

The final cultures were then subjected to centrifugation at about 3,000 r.p.m. for 15 minutes, after which the supernatant fluids were filtered through sintered glass. The filtrates were then tested for the presence of Brucella bacteriophage in the following way. One plate of well-dried S.D. medium was divided into four sections and each of them was flooded with a 36 hours' old broth culture of the standard strains of Br. abortus, No. S.19 and No. 544, Br. melitensis, No. 16M, and Br. suis, No. 5061, respectively. After the plate had been dried off in the incubator, all four sections were sub-divided into four parts, on each of which was deposited a loopful of one of the 37 filtrates and marked accordingly. The plate was left at room temperature until the drops had dried and then incubated for 48 hours at 37°C. in an atmosphere containing 10 per cent carbon dioxide when it was examined for evidence of lysis as was done in the first experiment. By that method, it was possible simultaneously to test four of the filtrates on the four standard strains of Brucella on one plate of S.D. medium. The remaining filtrates were tested for the presence of Brucella phage in the same manner.

(3) Small pieces from all of the 37 old cultures of Brucella on serum dextrose agar were added to 10 ml. of the broth Culture A. The resulting preparation was incubated aerobically at 37°C. for 48 hours and, after it had been stored at 6° - 9°C. for a further 24 hours,

hours/

a few drops were returned to broth Culture A. That procedure was repeated ten times. Each preparation was centrifuged and the supernatant filtered and tested for the presence of Brucella bacteriophage as in the second experiment. In the course of the test on preparation No. 5, there was observed one very small area of lysis which was of rectangular shape and measured one millimeter in breadth and two millimeters in length. The clear portion was excised and transferred to a bottle containing 5 ml. of serum dextrose broth, marked with the letter " K " and incubated at 37°C. for 48 hours aerobically. After centrifugation for 10 minutes at 3000 r.p.m., the supernatant fluid was filtered and tested for phage activity on the four standard strains of Brucella. With the aid of Br. abortus No. S.19, the " K " phage liquid was titrated and routine test dilutions used to determine the susceptibility of the 37 Brucella cultures under test in the manner that was described in Part 2. The search for the Brucella phage in preparation No. 5 and in nine others in the course of the experiment was repeated several times and the entire experiment was done three times in all.

(4) Here experiment No. 3 was duplicated with broth culture B in place of culture A.

(5) According to Adams (1959) coliphage, T_4, is activated by L-tryptophane which, in concentrations above 100 milligram per ml. in broth, activates the phage particles independently of temperature.

temperature./

At a concentration below 100 milligram per ml. the phage activity declines very rapidly with decreasing temperature.

L-tryptophane in concentration of 120 milligrams per ml. was added to serum dextrose broth and experiments Nos. 2, 3, and 4 were carried out again. For subsequent investigation, the broth was made to contain L-tryptophane to like concentration.

(6) From the 8 months old Brucella broth cultures which were studied for cell viability as described during the second part of the thesis, there was prepared a mixture consisting of a few drops of each of the 34 cultures, to which was added a broth culture composed of Culture B plus L-tryptophane. The final product was incubated at 37°C. for 48 hours aerobically and stored at 6°C, for 24 hours ere one ml. was returned to Culture B in tryptophane-broth. Once again, the procedure was repeated ten times. Each time the remaining fluid was tested for the presence of Brucella phage in the manner that has been described in the second experiment.

(7) Experiment No. 6 was repeated but without recourse to storage at low temperature.

(8) Ohashi (1939), quoted by Topley & Wilson (1955), claimed that phage more easily obtainable from the viscera than from the faeces or the intestinal mucosa of mice. In this experiment, small pieces of the spleens of Brucella infected guinea-pigs were added to a preparation

preparation/

in broth of Culture B plus L-tryptophane and the mixture was divided into two portions, one of which was subjected to the procedure of experiment (6) and the other to that of experiment (7).

3. RESULTS.

Cross-cultivation of the 37 cultures of Brucella, isolated from milk samples, with the four standard strains of Br. abortus (S.19 and 544), Br. melitensis (16M) and Br. suis (5061) failed to reveal the presence of Brucella bacteriophage.

Experiments No. 2, 4, 6, 7, and 8 also failed to yield evidence of active Brucella phage particles.

In the third experiment, one plaque was observed when preparation No. 5 was first examined for the presence of Brucella phage. The lysed area was detected in association with Br. abortus, No. S.19. After propagation, lytic activity was found to obtain with Br. abortus (S.19) and Br. abortus (544) but not with Br. melitensis (16M) or with Br. suis (5061). When phage liquid derived from Br. abortus (S.19) was titrated, routine test dilutions were found to lyse all the 37 Brucella cultures under study.

Meantime, preparation No. 5 and nine other serial ones forthcoming from the third experiment were re-examined several times for the presence of Brucella phage and experiment No. 3 was twice repeated in its entirety. In all those instances, lysis was not again

again/

encountered. Thus, the phage originally noted may have been due to particles derived from an external source or to contamination by one of the three stock phages.

4. DISCUSSION.

The results of this work suggest either (a) that none of the 37 Brucella cultures was lysogenic or (b) that all may have contained free phage particles so lacking in activity that they were unable to lyse any of the cultures to visible extent. Again, the cultures may have been lysogenic but did not contain free phage particles and were in need of induction. In a personal communication, Morgan (1962) informed me that he had been unable to demonstrate Brucella phage in either laboratory cultures or in manure obtained from Brucella-infected farms.

In the case of experiment No. 3, the writer is inclined to the opinion that the colonial abnormality encountered was not derived from the cultures under test for the following reasons:

(a) Had the area of lysis originated from the cultures, similar indications should have developed when the test was repeated;

(b) The rectangular form of the observed area of change was at variance with the circular shape of plaques usually associated

associated/

with individual phage particles;

(c) The lysis may have arisen from a drop of stock phage solution since, on the same day cultures of Brucella had been subjected to phage-typing and

(d) According to Lwoff (1953), "State preventing phage development conferred by the prophage to a lysogenic bacterium and allowing its survival after infection. A lysogenic bacterium is always immune towards the homologous phage - It may be immune also towards related phages". In reference to lysogeny and typing, Lwoff declared "It appears that immunity of lysogenic bacteria intervenes in lysotypy". If the altered area had been produced by a lysogenic strain, at least one of the cultures under test should have proved resistant to phage " K " whereas all the cultures were found to be susceptible.

The behaviour of phages, isolated from members of the genus Brucella, towards lysogenic strains of similar generic kind is scarcely mentioned by workers who have recovered lytic agents from cultures. The report by Parnas (1961) indicates that out of 164 strains of Br. abortus 63 proved resistant to the Tbilisi phage. The insusceptibility of some strains of Brucella to different phages has been recorded by Mamatsashvili (1957), whilst Morgan (1963) reported that of 3919 cultures of Brucella, the Br. abortus strains were susceptible to phage " Tb. ". As yet, the geographical distribution of lysogenic strains of Brucella appears to be confined to Russia and Poland. White (1937)

(1937)/

examined a number of strains of V. cholerae and found phage in all of those recovered in India but not in any that were of Chinese or of Japanese origin. The lack of accurate knowledge concerning the induction and inducibility of cultures calls for further investigation ere the matter of the geographical distribution of Brucella phage be finally determined.

5. SUMMARY.

(1) For the detection of Brucella phage in 37 laboratory cultures of Brucella and in five spleens from Brucella-infected guinea-pigs, eight different experiments were carried out.

(2) The methods used were designed to reveal lysogenic strains of Brucella that contained free phage particles.

(3) In seven of the experiments signs of lysis were not observed and, accordingly, the cultures were deemed to have been free from active phage particles.

(4) One plaque, encountered during the course of experiment No. 3, was subjected to propagation and tested for activity on four standard strains of Brucella as well as on the cultures under test. It was found to lyse all cultures except Br. melitensis (16M) and Br. suis (506).

(5) The origin of that solitary case of phagia was not finally determined but is believed to have associated with

with/

contamination by the stock phage solution used for typing cultures.

(6) It was finally concluded either: (a) that none of the 37 Brucella cultures was lysogenic, or (b) that all cultures were lysogenic and contained free phage particles of very low potency, or (c) that the cultures were lysogenic but lacked free phage particles and required to be induced.

GENERAL DISCUSSION.

The results of the first part of the investigation give some indication of the incidence of Brucella infection in the supply of milk available in the City of Glasgow and within a radius of 25 miles around. The information so acquired renders it possible to assess the prevalence of the disease in that part of Scotland and indicates that:

(1) 15.78 per cent of the attested herds involved in the production of certified milk conveyed live Brucella micro-organisms;

(2) 10.25 per cent of random samples of bulk raw milk destined for pasteurisation, contained the micro-organism;

(3) 23.21 per cent of abortions that occurred in 56 dairy cows were due to Brucella infection and

and/

(4) 4.98 per cent of the animals in one certified herd excreted Brucella micro-organism in the milk.

Those findings attest to an incidence of brucellosis in the West of Scotland higher than that in England, Wales and the North of Scotland. Mediarmid (1960a), for instance, has reported that 4.4 per cent of dairy herds in Oxfordshire and 2.2 per cent of those in the Isle of Man were infected by Brucella. An investigation conducted by 35 Public Health Laboratories in England and Wales (Anon, 1961) revealed that 4.8 per cent of the herds in the geographical areas involved were afflicted but the real incidence of herd infection throughout the country was deemed to be about 15 per cent. According to Marr and Williams (1958), in the North of Scotland about 6 per cent of the dairy herds proved to be affected by Brucella. The incidence of herd-infection revealed during the course of the investigation under report is almost three times that reported from other parts of Great Britain. Mediarmid (1961) has stated that the abortion-rate for dairy cattle in Britain is 2 per cent and that much of it is attributable to factors other than brucellosis. The results of the present study show that, at least, 23.21 per cent of 56 aborting animals were infected by Brucella. In an estimate of the losses due to Brucella infection in dairy cattle, Mediarmid (1960b) considered 10 per cent to be the average rate of infection for all the herds in

in/

England and Wales. On such a basis, the relative losses for the West of Scotland would be much higher. McDiarmid also reckoned on 5 infected animals for each infected herd with an annual loss of £25 per animal. The number of herds or of animals concerned in the production of Brucella-infected bulk milk involved in the present work is not known but, if McDiarmid's figures be applied only to the biologically positive samples encountered by the writer, 18 certified herds of the first group, 13 aborting animals of the third group and 5 infected animals of the fourth group would be involved so that the total monetary loss is likely to be:

18 x 5 + 13 + 5 = 108 animals £25 = £2700 per annum.

Not less momentous are the hazards which include (a) the danger to human beings from consumption of milk containing Brucellae and from the handling of infective bulk milk on farms as well as in pasteurizing plants, and (b) the risk to animals of infection from the milk and the discharges of both aborting animals and unrecognized affected stock. Kerr et al. (1958) and Kerr & Rankin (1959) have shown that infection by Brucella is readily established during the process of milking, whether by machine or by hand, and that in many vaccinated herds with little, if any, uterine involvement there is often a considerable incidence of infection of the udder. In vaccinated herds, too, infection may be of latent type that is not readily discoverable by either the owner or the veterinary practitioner. Such

Such/

animals constitute reservoirs of infection not only for other susceptible stock but also for human beings. Busch & Krüger (1961), on six occasions, took scrapings from beneath the finger-nails of a veterinary practitioner ten minutes to one hour after he had attended a cow that had aborted as a result of brucellosis and had at once washed his hands thoroughly. In two instances, guinea-pigs inoculated with subungual material developed brucellar antibodies to significant titre. Although the diagnosis of the disease in man is not always easy, some literature concerning the prevalence of acute and chronic brucellosis in the human population of Great Britain has been published. The most recent account is by Bothwell (1963 a,b & c), whose review (1963a) indicates that 45 fatal cases have been reported and emphasizes that much greater attention should be paid to the problem. That need together with the necessity for new anti-brucellosis regulations is also pointed out by Bothwell *et al.* (1962) and by Parry (1963).

One case of human brucellosis has been here recorded and full information regarding the diagnosis of 11 cases of human brucellosis since 1952 were kindly provided by Dr. W.J. Patterson, Medical Officer for the Eastern division of Glasgow. During the period, 1962-63, sixteen cases were recorded by Dr. Joan Landsman at the Department of Infectious Disease, Ruchill Hospital, Glasgow. Two other cases were also reported to me, one by Dr. Neil Reid, Medical

Medical/

Officer for Stirling, and another by Dr. Monro, County Medical Officer for Lanark. Table 18 shows the number of cases of human brucellosis that were diagnosed during the period, 1952 to 1963, in Glasgow and the Counties of Stirling, Lanark and Renfrew:

Table 18. Confirmed cases of human brucellosis in Glasgow and adjacent counties.

YEAR	CASES NOS.		TOTAL
	Male	Female	
1952	1	2	3
1953	1	-	1
1956	1	1	2
1959	-	1	1
1962	10	1	11
1963	11	2	13
Total	24	7	31

Of the above cases, seventeen occurred in the City of Glasgow and the remaining fourteen were detected in the counties beyond. One of the cases recorded by Dr. Patterson was traced to an attested herd from which the writer himself had recovered Brucella abortus. It is also noteworthy that in 1962 Dr. Patterson recorded the histories of 24 persons, belonging to five families, who had consumed raw milk derived from a dairy farm. Sixteen members of the group were found to have a normal serum-titre to Br. abortus. Of the remaining eight persons, one was ill and had a serum-titre of 1 in 8000 and seven

seven/

manifested titres of from 1 in 125 to 1 in 5000 without any sign of indisposition. Table No.18 further shows that an appreciably higher incidence of cases of human brucellosis occurred during the years 1962 and 1963, which increase may be due to the greater attention now paid to diagnosis of the disease among the human population. It may well be that the real incidence of human brucellosis in the West of Scotland is higher than the available evidence suggests and some statistics suggest that many cases are not diagnosed or are not notified. There are other problems which call for urgent investigation, such as the case of hospitalization and treatment of afflicted people and the loss of work involved. Quite complementary to that study is the determination of the incidence of brucellosis in the animal population.

Until 1940 brucellosis in Great Britain was believed to be due only to Br. abortus but, in that year Menton isolated from cow's milk a strain which possessed the serological and the biochemical characters of Br. melitensis. Since then many other similar strains have been encountered in this country. The types of Brucella isolated during this investigation included not only the classical Br. abortus but also dye-sensitive and aerobic strains of that species and strains similar to those recorded by Menton. Similar strains have been isolated from milk in England and Wales by Cruickshank (1954), by Stableforth (1960) and by various Public Health workers (Anon, 1961).

(Anon,1961)./

Stableforth (1960) reported that cultures of Brucella with the serological and biochemical characters of melitensis have been isolated from cattle in Britain but were not any more pathogenic than the typical Br. abortus. In relation to human infection, those micro-organisms resembled Danish strains of Br. suis in that they seemed to be less pathogenic, and that, although until 1955, thirteen outbreaks had been diagnosed, but human infection had not been recorded. The oxidative metabolic pattern and the susceptibility of those strains to Brucella bacteriophage also differs from that of typical strains of Br. melitensis (Meyer & Morgan 1962) and (Meyer, 1962). In certain literature, those strains have been reported to as British melitensis, but Jones (1960) has reported the isolation of similar strains from cattle of Wisconsin. Wundt (1962), too, has recorded that of 17 strains of Br. melitensis isolated in Northern Germany 13 were susceptible to 8 Brucella phages, whereas of 29 Br. melitensis strains recovered in Western Germany only one proved liable to the effect of phage. The pathogenicity of the strains was not mentioned.

As yet, the isolation of active Brucella bacteriophage from Brucella cultures or infected materials has not been demonstrated from countries other than Russia and Poland. During the present experiment, attempts to demonstrate the presence of Brucella phage in 37 cultures of Brucella were unsuccessful.

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THE INCIDENCE OF BRUCELLAR INFECTION IN THE WEST OF SCOTLAND WITH SPECIAL REFERENCE TO THE MILK SUPPLY OF THE CITY OF GLASGOW.

A THESIS

submitted by

R. A. TADAYON, D.V.M. (TEHERAN)

for the Degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow.

SUMMARY.

A total of 380 bovine milk samples were obtained, namely:

(a) 128 mixed samples from 114 farms suppling certified milk to the City of Glasgow and its environs;

(b) 128 mixed samples from 27 pasteurizing plants in the City of Glasgow. Seventy-eight of those were bulk raw milk and the remaining 50 were pasteurized milk;

(c) 73 samples of milk from 56 individual aborting animals on various farms in different parts of the West of Scotland and

(d) 51 samples of certified milk from as many cows on one farm associated with a case of human brucellosis.

The above specimens were subjected to the milk ring test, the milk plate agglutination test, the milk capillary tube test, the milk whey tube agglutination test as well as biological, cultural and microscopical examinations.

Of the 114 originall samples concerned in the first group:

23	(20.18 per cent)	were positive to the milk	ring test,
20	(17.54 do.)	do.	plate test,
19	(16.67 do.)	do.	capillary tube
10	(8.77 do.)	do.	whey tube test,
18	(15.78 do.)	do.	biological test and
1	(0.87 do.)	do.	cultural examination

Of 87 bulk raw samples in the second group:

group:/
39 (50.0 per cent) were positive to the milk ring test,
19 (24.4 do.) do. plate test,
21 (26.9 do.) do. capillary tube t
14 (17.9 do.) do. whey tube test a
8 (10.26 do.) do. biological test.
Of 50 pasteurized samples in that group:
29 (58.0 per cent) were positive to the milk ring test,
15 (30.0 do.) do. plate test,
14 (28.0 do.) do. capillary tube t
11 (22.0 do.) do. whey tube test a
none to the biological and cultural examination.

Of 56 samples of milk from individual aborting cows in the third group:
24 (44.44 per cent) were positive to the milk ring test,
23 (41.1 do.) do. plate test,
27 (48.1 do.) do. capillary tube
22 (39.3 do.) do. whey tube test,
13 (23.21 do.) do. biological test,
1 (1.78 do.) do. cultural examination

Of 51 samples of certified milk from individual cows in the fourth group:
10 (19.6 per cent) were positive to the milk ring test,
9 (17.7 do.) do. plate test,
9 (17.7 do.) do. capillary tube t
8 (15.7 do.) do. whey tube test,
5 (9.8 do.) do. biological test and
3 (5.88 do.) do. cultural examination.

The worth of the milk ring test, modified by means of centrifugation, appeared to exceed that of the standard incubation method.

It was noted that pasteurization altered the size of the fat globules of milk and consequently affected the outcome of the ring test.

test./

Forty-eight cultures of Brucella were typed by means of conventional biochemical and serological methods and by use of Brucella bacteriophage.

Study of cell viability and dissociation in 34 of the Brucella cultures originating from smooth colonies and stored at 6°- 9°C. indicated that, (a) in broth up to 5 per cent of viable cells survived for as long as eight months and (b) from 10 to 75 per cent of variant cells were demonstrable among the smooth population after a lapse of 8 months

For the detection of Brucella phage in laboratory cultures of Brucella eight different experiments were carried out.

The incidence of brucellosis in the West of Scotland proved to be about three times higher than that so far recorded for England, Wales and the North of Scotland.