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A SURVEY AND BACTERIOLOGICAL STUDY OF BRUCELLOSIS IN SELECTED PARTS OF LEBANON AND SYRIA.

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ABSTRACT

A SURVEY AND BACTERIOLOGICAL STUDY OF BRUCELLOSIS IN SELECTED PARTS OF LEBANON AND SYRIA.

1. A historical review tracing the development of the problem of Brucellosis from its appearance in the literature till the present day was presented.

2. A survey of goat's milk, cow's milk, goat's serum and human serum, obtained from selected representative areas of Lebanon and Syria was made for the presence of B. melitensis, B. abortus and their specific agglutinins. Strains of B. melitensis and B. abortus were isolated and their cultural characteristics investigated.

3. A total of 1539 milk specimens were examined of which 269 came from Syrian goats and 1270 came from either cows or goats of Lebanon.

4. The average incidence of infection among animals in Syria and Lebanon as judged from the number of positive findings of B. melitensis and B. abortus in goat's and cow's milk was 0.91 per cent. This reflects the relative incidence of active infection. The incidence of past infection among the animals as judged by the positive titer obtained from whey agglutination tests was 17.3 per cent. The current incidence of infection in cows and goats were of relatively similar magnitude, being 0.85 and 0.93 per cent respectively. Past infections as indicated by whey agglutination tests also were relatively equal for bovine and caprine strains, the

incidences being 18.8 and 16.6 per cent respectively. Goat sera agglutination tests, on the other hand, indicated a much higher incidence of past infection among the goats to the extent of 67 per cent. Cow sera were not available for study but a survey of 641 human sera from the A.U.B. Hospitals gave an incidence of six per cent of positive agglutination tests for B. abortus and B. melitensis.

5. The average incidence of current infections for goats from both Lebanon and Syria was 0.93 per cent. Infections were about twice as frequent in the Syrian goats (1.5 per cent) however, as in the Lebanese goats (0.74 per cent). Infection in Lebanese goats was higher in Tripoli (2.5 per cent) than at Laklouk (1.05 per cent) and least in Beirut (0.38 per cent). The incidence of current infections among Lebanese cows was 0.85 per cent but a higher rate of infection was found in Mashghara area (1.25 per cent) than in Beirut area (0.65 per cent).

6. The average incidence of past infections for goats from both countries was 16.6 per cent. The incidence of past infections was 18.2 per cent for the Syrian goats and 16.1 per cent for the Lebanese goats. The incidence was higher in the Tripoli area (25.6 per cent) than in the Laklouk and Beirut areas (15.8 per cent) and (15 per cent) respectively. The incidence of past infections among Lebanese cows was 18.8 per cent. This figure was higher in Mashghara (22.2 per cent) than in Beirut area (17 per cent).

7. The cultural characteristics of the B. melitensis and B. abortus strains isolated from Lebanese and Syrian goats and cows, agreed essentially with the description given for these organisms by previous workers.

HISTORICAL BACKGROUND

BRUCELLA MELITENSIS

Undulant fever was recognized as a clinical entity by Marston in 1859 (116). It took a real place in medical history when it swept through the ranks of the British Army during the Crimean War, breaking out on the Island of Malta among soldiers who drank raw goat's milk. Many authors suggested that this disease, like many others, may well date as far back as the time of Hippocrates (about 450 B.C.), who described a fever which historians now recognize as being Malta fever. During the 18th and 19th centuries various accounts of the disease appeared and during the Crimean War considerable confusion existed due to the simultaneous presence of Malta and Typhoid fevers in the British troops.

In 1886, Sir David Bruce (13) was the first to establish the etiology of this disease by describing a small cocco-bacillus seen in sections of the spleen of fatal human cases. The following year, he isolated the bacillus from human cases on the Island of Malta.

Hughes (89) in 1892 named the causative organism Streptococcus melitensis, while Bruce (14), a year later, called it Micrococcus melitensis. Early workers, obviously, did not recognize the bacillary form of the organism.

The disease was given many descriptive names after its locality, nature or course among which were Mediterranean fever, goat fever and dust fever. It was Dr. M. L. Hughes (90) of the British Army who in 1897 named it "Undulant Fever" after the

remissions and recurrences of the fever associated with it.

By 1904 the disease had become quite a problem in Malta, so a commission was appointed by the British Admiralty, the War Office and the Civil Government of Malta to study it. This commission established the fact that over 40 percent of the goats on the island of Malta were infected. Hughes, however, never had the suspicion that the origin of the infection in human beings lay in goats' or cows' milk, but was under the impression that it was in the sewage. In 1905 Zammit (185), a member of the British Commission, was the first to discover that a big percentage of the infected goats eliminated virulent bacteria in their milk and thus established goat milk to be the chief host of Brucella melitensis.

Human cases were traced to drinking infected milk. Many other cases were found in herdsmen and those handling the infected animals. Many of these were found to have been infected through the skin abrasions contaminated with urine from infected goats, in which large numbers of organisms were found.

In the Commission's final report they stated that the most common method of infection in man was by the ingestion of infective articles of food, mainly milk.

Brucella melitensis localizes in the udder, spleen and lymph nodes of the goat, giving rise to an interstitial mastitis and splenic lymphadenitis. The organism has also been recovered by Shaw (159) and Duke (33) from the milk of cows living in contact with goats and from fetuses of sheep and goats.

BRUCELLA ABORTUS

In 1897 Bang (6) in Denmark isolated a micro-organism responsible for a contagious abortion in cattle, which he termed Bacillus abortus. The disease is now recognized as "Bang's Abortion Disease".

In the cow Bacillus abortus localizes primarily in the pregnant uterus, more particularly in the fetal membranes (chorion) and secondarily in the udder ducts. The pregnant cow shows no clinical manifestations and the infected udder presents no signs of mastitis. The pathologic picture of the chorionic disease is the invasion of the epithelium by the bacillus, and its multiplication therein (161). In the cow's udder the organisms, escaping into it from the circulation, are probably retained in the residual milk from day to day and multiply there.

Brucella abortus has been found in animals in all parts of the world. It has been recovered from naturally infected horses, fowls, dogs, sheep, wild deer, wild buffalo and human beings.

In 1894 Theobald Smith first observed a peculiar disease occurring in guinea pigs following their inoculation with milk, that simulated tuberculosis. It was only in 1911 that he and Fabyan (163) determined definitely that these lesions were caused by Brucella abortus.

In 1911 Schroeder and Cotton (152) asserted that the udder was the habitat of choice of Brucella abortus, in fact the only habitat in the non-pregnant cow. One animal in their work showed bacilli in her milk for seven years. These same workers called attention to the fact that milkers may perpetuate the disease by

carrying it on their hands.

In 1913 Zwick and Krage (188) isolated organisms from the milk of three cows. They noted no changes in the udders or milk microscopically and concluded that Brucella abortus may pass through the udder without causing lesions.

In 1913 Schroeder (151) fed guinea pigs with infected raw, pasteurized and boiled milk and said that several of the guinea pigs receiving the raw milk developed abortion disease.

In 1917 Fleichner and Meyer (52) concluded from inoculating guinea pigs with certified milk that Bacterium abortus was for all practical purposes, always present in certified milk of infected cows.

Alice C. Evans (39) deserves by shear merit the credit of the most comprehensive purely bacteriologic work that has been done on milk. She well deserves the credit for being the first one to notice the very intimate relationship existing between Micrococcus melitensis and Bacillus abortus and for the suggestion that the latter organism may be pathogenic for man.

Evan's findings were confirmed by many observers including Meyer and Shaw (129) who studied 21 strains of Brucella melitensis and 32 strains of Brucella abortus and found that these two types cannot be distinguished from each other on morphological, cultural or fermentation grounds. They suggested the generic name Brucella to separate and distinguish them from the genus Bacterium.

BRUCELLA SUIS

The recognition of porcine abortion in America by Traum (172) in 1914 was approximately 17 years after Bang described the

cause of infectious abortion in cattle. His report, as well as those of many other investigators, indicate that the porcine cultures are more virulent for man than is the bovine type and the handling of infected material leads to infection more readily than does the ingestion of contaminated food. Hence, packing-house employees who handle hog products work under conditions peculiarly hazardous for contracting brucellosis (42). The results of the several investigators have agreed in showing that individuals who come in contact with infective material may develop immunity reactions without suffering any notable illness.

METABOLIC ACTIVITIES OF THE BRUCELLA

The following presentation is a brief review of the literature that deals with the metabolic activities and nutritional requirements of Brucella.

The nutritive requirements of Brucella are relatively complex and the best growth is obtained on enriched media. In an attempt to prepare a synthetic medium that would support the growth requirements of Brucella in a chemically defined medium have been studied by Zobell and Meyer (187-VIII). Lactates, citrates, cystine, asparagine and other compounds supply to a lesser degree the carbon or energy requirements. The ammonium ion and certain amino-acids furnish the nitrogen. The minute sulphur demands are satisfied by sulphates and thio-amino-acids. Phosphates suffice for the indispensable phosphorus. Of the mineral elements, magnesium and little sodium or potassium are essential and iron is beneficial. In the absence of knowledge of the accessory growth

factors required by this group, only slight growth was obtained when such media were used. McNutt and Purwin (123) found that when Brucella was limited to a sugar as the only source of carbon, growth took place in both arabinose and xylose. It was believed that in these instances ingredients were used which are known to carry various accessory growth factor substances as contaminants.

Koser, Breslove and Dorfman (108) were the first workers to obtain luxuriant growth of the Brucella in a chemically defined medium by the addition of known accessory growth factors. The medium consisted of 17 amino-acids, glucose, inorganic salts and the appropriate accessory factors: nicotinamide, thiamin, pantothenic acid and biotin concentrate. Koser and Wright (109) substituted the biotin concentrate with pure biotin or biotin methyl ester and obtained similar results.

McCullough and Dick (121) determined the qualitative and quantitative requirements of the accessory growth factors for some laboratory strains of Brucella and confirmed the findings of Koser et al. Thiamin 0.2 , nicotinic acid 0.2 , calcium pantothenate 0.04 and biotin 0.001 per cc. of medium were required for use in accessory factor tests. They found that strains requiring an increased carbon dioxide tension were not successfully grown in this medium, but after acclimatization to atmospheric conditions growth of 30 strains was obtained.

In 1943 McCullough and Dick (122) and Schuhardt and Beal (153) using a glucose-inorganic salt medium, found that Brucella melitensis and Brucella suis required the presence of thiamin and nicotinic acid amide while Brucella abortus required the presence

of biotin in addition to thiamin and nicotinic acid amide. These growth factor requirements are the same as those reported by Koser et al. who used an amino-acid medium. Pantothenic acid while not indispensable enhanced the growth of most of the strains.

The failure of Brucella to develop in media of known chemical composition in the absence of accessory growth factors, presents a decided contrast to the prompt growth in infusions of meat, liver and yeast. These sources contain something necessary for growth. Preliminary studies have shown that the growth-promoting principles in tissues and yeasts may be separated to some extent from other materials and partially purified (144),(150). Kerby (101) reported that addition of nicotonic acid and thiamin to Bacto-tryptose agar enhanced the size of the colonies of several strains of Brucella abortus. Since these two accessory factors were added to a peptone medium which in itself supported good growth of Brucella, it was not clear whether the accessories were needed for cell multiplication.

Of the sundry media that have been so far developed for Brucella cultivation, none is absolutely satisfactory. With liver infusion agar it was observed that many times if a given inoculum will yield 600 or 650 colonies on a pour plate, an inoculum half as large will yield absolutely no colonies (102). The Bacto-tryptose medium introduced by the Difco Laboratories, Detroit, has the advantage of giving a quicker initial growth. Liver-infusion medium prepared according to the method of Hasley and Schalter (75) was not as satisfactory for the cultivation of Brucella as is tryptose broth. A milk-tryptose-crystal violet medium described by Kerby

and Calder (102) was proved to be superior to liver infusion broth or tryptose broth in primary culture.

In 1939 Metzger, Beaudette and Stokes (125) obtained pure cultures of Brucella abortus by inoculating the cream and sediment of milk into the chorio-allantoic membrane of eggs on the tenth day of incubation. Such cultures contained small numbers of these organisms and large numbers of udder streptococci.

DIFFERENTIATION OF THE MEMBERS OF THE GENUS BRUCELLA

The agglutinin-absorption test was the first method used to establish differences between the members of the genus Brucella. In 1920 Feusier and Meyer (50) who were the first to contribute data on this subject identified four types. Later, Khalid in 1921 (103), Burnet in 1922 (16), Evans in 1923 (41) and Ross in 1927 (147) identified many different types, but the most inclusive work was done by Evans. She studied 68 strains of Brucella of animal and human origin, coming from all parts of the world, and established two main serological types. Smooth strains of Brucella abortus and Brucella suis constituted one main type and smooth strains of Brucella melitensis constituted the other. Wilson and Miles in 1932 (177) showed that these two main types are distinguished not by qualitative different antigens, but by a quantitative distribution of a common antigen. Brucella melitensis contains a large amount of M and a small amount of A antigens, while both Brucella Abortus and Brucella suis contain large amount of A and small amount of M antigens. The A:M ratios in abortus and in melitensis are assumed to be about 20:1 and 1:20 respectively.

It was impossible by the agglutinin-absorption test to separate Brucella suis and Brucella abortus. Furthermore, some strains of Brucella possessing antigenic variant characteristics could not be easily classified as to species.

The pathogenicity tests proposed by Smith (162) and Carpenter (18) in 1926 for differentiation of species have certain limitations. They pointed out independently by infecting guinea pigs with minute doses of recent strains that porcine variety can be distinguished from the bovine variety of Brucella abortus by the character of the more chronic lesions resulting particularly in the spleen and lymph nodes.

In 1928 and 1929 two methods were proposed for the differentiation of the varieties of the genus Brucella. The first, developed by McAlpine and Slanetz (119), depends on certain differences in glucose utilization. The abortus-melitensis group is divisible by this test into two divisions. The first known as the abortus group, made up of strains that are incapable of utilizing more than two per cent glucose in the presence of Fairchild's peptone and consequently demize the amino-acids and render the medium increasingly alkaline; and the second group which includes the porcine and melitensis strains that hydrolyze from five to twenty percent of glucose under the same conditions. These glucose metabolism studies, however, do not furnish distinct differences in the varieties of the genus Brucella, for quite a number of the abortus cultures utilize more than two per cent, while several melitensis strains split the carbohydrates in amounts below five per cent. Moreover, McAlpine, Plastridge and Brigham

(118) showed that strains growing for several generations, lose their ability to utilize dextrose, but upon transfers in liver infusion broth with continued incubation at 37°C. for two weeks, these strains developed a new form (mucoid) in which the glucose utilizing power was restored. McAlpine and Slanetz (119) found that these differences in the metabolism are closely paralleled by the behavior of the strains toward carbon dioxide. Thus the growth of the abortus cultures is accelerated by the cultivation in an environment containing five to ten per cent carbon dioxide, while the porcine and melitensis cultures are more or less inhibited when inoculated under the same conditions. These procedures fail to distinguish between the porcine and melitensis strains.

The second method, recommended by Huddleson (81), determines the growth differences of the species by the use of dye media. He divided the group Brucella into three varieties:

1. Brucella abortus, thionin genesistatic, fuchsin and methyl violet fast.
2. Brucella suis, thionin fast but fuchsin and methyl violet genesistatic.
3. Brucella melitensis, thionin, fuchsin and as a rule, methyl violet fast.

However, there are some difficulties in the dye plate method of differentiation. Occasionally, a batch of liver agar would reduce a dye spontaneously, especially thionin, thereby rendering it unfit for use. This characteristic has been attributed by Meyer and Zobell (187) to various substances, and combination of substances. They stated that "Variation in peptone and protein content

of the basic medium affects the toxicity of the dyes. The presence of reducing substances, such as glutathione, sulphydryl group and respiratory pigments activate the transformation of the dye molecules. The age of the liver, the method of extraction, filtration, etc., materially influence the composition of the basic medium".

In 1929 Huddleson has supplemented the shortcomings of the previously mentioned tests by a study of the rate of hydrogen sulphide production and then by a comparison of the genesistatic properties of certain dyes to interfere with the reproductive mechanism of the representatives of the genus Brucella. On a favorable medium, Brucella suis produces hydrogen sulphide for at least six or seven days. Brucella abortus produces hydrogen sulphide for only one or two days and Brucella melitensis does not produce an appreciable amount at any time.

Kristensen (110) and Thomsen (169) reported that the Danish porcine strains give negative hydrogen sulphide. Furthermore, they found that under identical conditions typical abortus strains may fail to generate hydrogen sulphide, while melitensis strains may produce small amounts of the gas. From these results Zobell and Meyer (187-V) concluded that too much reliance cannot be placed on the hydrogen sulphide test in the classification of individual strains.

The ability of Brucella to reduce nitrates is a significant and valuable differential characteristic. Evans in 1918 (40) showed that some cultures have a slight tendency to reduce nitrates

to nitrites irrespective of being Brucella abortus or Brucella melitensis. Duncan and Whitby in 1930 (34) and Bergey in 1931 (8) described the whole Brucella group as non-reducers. Topley and Wilson (170) reported that nitrates are occasionally reduced by certain strains. Zobell and Meyer (187-VI) asserted that Brucella reduce nitrates to nitrites when tested in a semi-solid medium containing 0.2 per cent potassium nitrate. The nitrites are also rapidly reduced. These observers were also able to find a sufficient difference in the nitrate and nitrite-reducing power of the three species of Brucella. "Suis strains destroy 0.05 per cent potassium nitrite in five days, while the abortus and melitensis types lack this ability. The melitensis varieties are in general more active reducers of nitrites than the abortus". It should always be borne in mind that none of the various tests for the differentiation of the genus Brucella is reliable alone. In fact, it is advisable to use as many tests as possible in an attempt to differentiate one species from the other.

DIAGNOSIS OF UNDULANT FEVER

The diagnosis of Undulant fever is often very difficult. In many cases, the symptomatology is either indefinite or may resemble that of other diseases. The great diversity of signs and symptoms produced by this infection compels the clinician to rely upon the laboratory for diagnosis. There are many different laboratory tests which will be discussed in the order of their decreasing importance.

Cultural Test:

Isolation of Brucella from the blood, stool or urine (182), (64) stands first as a diagnostic aid in confirming the clinical diagnosis of Brucellosis. Such isolation, moreover, when supplemented by the Huddleson dye method, or any other differential test, reveals the species of organism and is of great value in establishing the animal source of infection. The point against it is that besides the inherent technical difficulties, blood, stool and urine culture take too long for an ordinary diagnostic or public health report.

Agglutination Test:

The agglutination test, because of its ease in performance and its specificity, is the reaction of choice in most laboratories. Here the matter of technic, which involves the choice of strain to be used, the preparation of the antigen, the time of incubation and other details, is of extreme importance. The question of the proper titer to be obtained before a positive diagnosis can be made is a perplexing one. Some workers think that any case showing a titer of 1:80 or above should be considered diagnostic (77), and that titers of less than 1:80 have little or no significance. On the other hand, even in severe cases in which Brucella infection is proved by cultivation of the organism, skin tests and animal inoculation, agglutinins may be lacking in the serum. That was true in Evans' case (42) and Carpenter, Boak and Chapman (23), who have recognized severe cases presenting a weak titer of agglutinins or none in the serum. Burnet (15-III), (16) reported that 16.6 per cent of his cases failed to show agglutinins in the serum

and Simpson (155) reported a number of such cases. Infection without the occurrence of agglutinins in the serum would be even more likely to occur in mild (chronic) than in severe (acute) cases (42). Hence, a negative agglutinin reaction cannot be regarded as significant in chronic cases and a low titer of 1:10 or over is now being stressed in the diagnosis of the ambulatory cases (155),(74). Furthermore, the agglutination test may be positive, in dilutions of 1:80 or more, with or without any evidence of clinically active Brucellosis (117),(94),(28),(124),(84). Therefore, whenever the results of any agglutination test are considered, the question of past or present infection becomes of importance. Carpenter and Boak (22) are of the opinion that the agglutinins usually remain for a long time; hence, in the interpretation of positive agglutination reports, the persistence of reacting bodies from a previous infection must always be considered.

As Brucellas have certain antigenic relationships with other organisms, it is worthwhile to mention those for they should be kept in mind while running an agglutination test.

Since first reported by Francis and Evans in 1926 (57), it has become widely recognized that the serum of patients suffering from tularaemia and Brucellosis may have cross agglutinins. On the other hand, such cross agglutinations may also occur between Brucella and Proteus. Nicolle and Comte (135) observed the agglutination of Brucella melitensis and Brucella abortus by typhus serum. They noticed that the agglutinins for Brucella disappeared within two days, at the time when the Weil-Felix reaction developed. Kemp et al. (100) reported four cases of Undulant fever in which

various Proteus strains were agglutinated in titers ranging from 1:80 to 1:240, but stressed the important fact that there was no increase in the titer as the disease progressed. In 1941 Calder (17) found high agglutinin titers for Proteus in chronically febrile patients who gave no clinical history or physical signs suggesting the possibility of Typhus or Rocky Mountain Spotted Fever.

In 1946 Eisele, McCullough, Beal and Burrows (35) demonstrated the development of Brucella agglutinins in humans following vaccination for cholera. In 1947 (36) they found that in hundred subjects who had been vaccinated against cholera while in military service, 56 per cent had positive Brucella agglutination reactions in titers of 1:20 or higher, 41 per cent had titers of 1:40 or higher and 20 per cent had titers of 1:80 or 1:160. In the group who were tested 18 or 28 months after vaccination, 27 per cent still had titers of 1:40 or higher.

Skin Sensitivity:

With the exception of a very few observers who explain the phenomenon as an indication of immunity or a phase of anaphylaxis, cutaneous hypersensitiveness is now generally considered to be an evidence of infection. Fleichner, Meyer and Shaw (54) after a series of experiments on guinea pigs, came to the conclusion that by repeated injection of dead Brucella abortus or abortus protein into guinea pigs, it is possible to develop a high grade of immunity as evidenced by the marked agglutinin content of the blood. Incidental to the development of this immunity, these animals became highly sensitized and some of them developed a pronounced

anaphylactic state. Cutaneous hypersensitiveness was never present. By subsequently injecting them with living organisms, skin tests were always present if on postmortem examinations there were pathologic evidences of infection. A state of an anaphylactic hypersensitiveness can exist without the least cutaneous hypersensitiveness. Stroem in 1931 (167) found that cutaneous hypersensitiveness was present in all animals infected with living Brucella, even when pathologic lesions were absent on gross examination. The difference between Stroem's and Fleichner's results might be due to the fact that Stroem strains were old laboratory strains that seemed to have lost much of their ability to cause gross lesions, but were still able to sensitize the body.

In human beings cutaneous hypersensitivity to organisms of the genus Brucella was first investigated by Fleichner and Meyer (53). Using a saline suspension of the organisms, they tested 75 infants who had been fed milk containing Brucella abortus and found two with a specific skin sensitivity. Later, many other preparations were used as antigens. The broth filtrate used by Burnet (15-III), Trenti (174), Olmer and Massot (136), Dubois and Sollier (32), has been dispensed with because of the possibility of a pseudo-reaction (61); a saline suspension of the killed organisms used by Sensenich and Giordano (154), Giordano (61), Simpson and Frazier (157), Favorite and Culp (47), gives accurate and specific results but may cause a severe reaction, and is always followed by a positive agglutination test. Leavell and Amoss (112) claimed more specific reaction with heat-killed suspensions than with extracts. A single strain of Brucella melitensis group is suffi-

cient as an antigen in performing the test, since it has been found that other members of the group give similar results (184). Nucleoprotein fractions in saline, or fat-free alcohol extracts, have been used by Levin (113), Simpson (155), Henry and Traum (78), Giordano and Sensenich (62) and Huddleson, Johnson and Hamann (87). They probably give a much less general reaction, with equal specificity, but are usually followed by a positive agglutination test.

In 1934 Goldstein (63), Heathman (76) and Huddleson (84) demonstrated the appearance of agglutinins after skin testing. Evans in 1937 (44) and Menefee and Poston in 1939 (124) showed that the endermic injection of brucellergen causes a rise in the patient's opsonins and agglutinins, which reaches a peak near the end of the third week.

In 1938 and 1940 Morales-Otero and Gonzalez (132),(134) prepared a purified protein antigen from Brucella and used it to investigate cutaneous hypersensitiveness to Brucella among milkers and cattle handlers in farms. It was found (133) that contact with infected material causes the development of a state of hypersensitiveness, as shown by injecting the purified Brucella protein, although signs or symptoms of the disease were absent. This has been explained by the fact that hypersensitiveness usually appears later than agglutinins and complement-fixing antibodies and that it lasts longer.

Brucellergen prepared by Huddleson (84), was found superior to the other antigens, because it eliminates a certain percentage, but not all of the non-specific reactions. Angle in 1938 (2) and Keller in 1940 (98) found that brucellergen and heat-killed

vaccine of Brucella suis and Brucella abortus are equally effective, although the latter gave a higher incidence of local sloughs and systemic reactions.

The skin test has been used frequently for the diagnosis of Undulant fever (61),(113),(92),(87),(120),(63),(47),(99),(66),(124), (98) and also in surveys designed to evaluate the incidence of infection in various occupational groups, such as meat packers, veterinarians and laboratory workers (88),(76),(127). The diversity of preparations used as antigen makes correlation of results of investigators difficult. As a rule, a positive reaction has been considered as denoting past or present infection. The highest percentage of positive reactors in those groups closely associated with animal carcasses and persons with long periods of association with domestic animals, points strongly to animal contact as an important factor in the development of positive reactions. Certain observations have made the interpretation of the skin test difficult. For instance, some individuals from whose blood Brucella abortus has been cultivated, have been shown to have negative skin reactions (23),(30),(31),(168),(141),(43),(46). Moreover, consideration must be given to the fact that normal persons may develop cutaneous hypersensitiveness without having been aware of symptoms of illness as a result of Brucella infection (124), and that cutaneous hypersensitiveness persists for years after complete recovery. Therefore, the skin test may seem of little value in the diagnosis of the individual case, but further knowledge of the epidemiology and incidence of Brucellosis in man may be gained by its use on large groups.

Kolmer in 1940 (106) and Meyer in 1941 (127) found that previous vaccine injections did not induce positive intradermal reactions. A positive reaction, therefore, can be regarded as having the same significance as a positive tuberculin test in tuberculosis. It indicates past or present Brucella infection but bears no relationship to clinical entity.

Complement-Fixation Tests:

The value of the complement-fixation test for the diagnosis of Brucella infection in animals was indicated by Holth in 1909 (80). In 1913 Larson and Sedgwick (111) stated that 17 per cent of 425 children whom they had examined gave complement-fixation with Brucella abortus and that this reaction ran parallel with agglutination. Moore (131) also stated that the "complement-fixation test" is considered equally as good as the agglutination test, or more satisfactory. Zeissig and Mansfield (186) reported the results of their comparative studies of the agglutination and complement-fixation tests for the detection of Brucella abortus infection in cattle. They concluded that "the agglutination and complement-fixation tests" agree very closely in regard to the status of the animals under test. Carpenter and Boak (22) stated that the agglutination test is the simplest and most satisfactory method of ascertaining whether or not a patient has Undulant fever, and that the complement-fixation test has no advantages over the agglutination test. Sasano, Caldwell and Medlar (149) examined the sera of 1,000 persons for complement-fixation and agglutination and concluded that complement-fixing substances and agglutinins

develop about the same time and that complement-fixing substances persist longer than agglutinins.

In 1942 Wise and Craig (181) showed that because the appearance of complement-fixing antibodies precedes that of agglutinins, complement-fixation may be useful for the diagnosis of acute Brucellosis. They found out that brucellergen used in skin testing provokes the appearance of complement-fixing antibodies and, therefore, concluded that the finding of a positive Brucella complement-fixation reaction in individuals giving no history of an illness suggestive of Brucellosis and having never been skin tested or given vaccine, is considered to indicate contact with Brucella organisms but does not indicate active infection.

Opsono-Cytophagic Test:

In 1933 Huddleson and his associates (87) developed the opsono-cytophagic test, which is a modification of the opsonic index test. They recommended its use in conjunction with the skin reaction and stated that valuable information as to the status of the patient could be obtained if the results were properly interpreted. They showed that by use of citrated blood of humans who were known to have had Undulant fever in past years and shortly after recovery of those who are actively infected, or who have no history of the disease, that the in vitro activity of the polymorphonuclear cells in whole citrated blood for Brucella is an expression of immunity to Brucella and an indication of the progress toward recovery in active infection. The absence of, or a low phagocytic activity obtained in conjunction with a negative allergic

test is evidence of susceptibility to Brucella infection. A subject reacting positively to the intradermal test is classified as infected when less than 40 per cent of his polymorphonuclear leukocytes show marked phagocytosis and as infected but with questionable immunity if from 40 to 60 percent of his polymorphonuclears show marked phagocytosis. A subject reacting to the intradermal test is classified as immune when 60 per cent of his polymorphonuclears or more show marked phagocytosis of Brucella organisms (66). In 1940 Keller and his associates (98) corroborated the results of Huddleson and his co-workers. They indicated the specificity of the combined tests, intracutaneous and opsono-cytophagic, in individuals who have or have had Undulant fever, and the lack of response in those who have other febrile conditions.

In 1939 Evans (45) considered that the opsono-cytophagic test is the least reliable of the laboratory methods, and that a positive agglutination reaction is the most significant evidence of Brucella infection.

Conclusion:

Results drawn from the different studies of the laboratory methods show that, not a single investigator has completely agreed with the other on a definite procedure for the diagnosis of Malta fever. Since none of the tests mentioned for the diagnosis of Brucellosis gives results that may be interpreted as positive or negative evidence of Brucellosis, a consideration of the combined results of all the tests is the best procedure. Recognition of chronic cases remains a clinical problem using the laboratory procedures available as aids rather than as determining factors.

BRUCELLA IN MILK AND MILK PRODUCTS

The seriousness of the problem of Undulant fever necessitated a thorough and careful study of the sources of infection. The results of these long and elaborate studies established two routes of infection:

1. Through the skin, particularly the abraded skin.
2. Through the portal of the alimentary canal.

The second portal of entry indicated the need for examining all those foods in which Brucella naturally occur and this fact had been instrumental in directing the trend of work of the early investigators.

Zammit in 1905 (185) and later many other investigators, have demonstrated beyond question, the fact that a high percentage of infected goats eliminate Brucella melitensis in their milk.

Smith and Fabyan (163) and Schroeder and Cotton (152) were able to infect guinea pigs by injecting into them milk from infected cows and thus demonstrated the presence of Brucella abortus in that milk. Thus both organisms, Brucella melitensis and Brucella abortus, were found in milk coming from infected animals.

The number of Brucella discharged daily in the milk is very variable but for the most part, the greatest numbers are present soon after abortion. The results of the Mediterranean Fever Commission (1907) on the number of Brucella melitensis organisms present in goat's milk each day showed a variation from none to 30,000 organisms per cubic centimeter.

The infection in certain animals will become greatly reduced or diaappear in three or four weeks, while in others, it may persist

throughout a lifetime. Jones (93) studied the seasonal variation in the incidence of Brucella abortus in raw milk. It was found that the number of positives reached a maximum about January and a minimum about August.

Evans (38) after examining milk from two cows that had been artificially infected many times with Brucella abortus, concluded that virulent strains of the organism were not eliminated continually in great numbers in milk from aborting cows. Carpenter and Boak in 1928 (21) found that a small number of organisms were present in the milk of naturally infected animals. Huddleson, Hasley, and Torrey (86) and Carpenter and Baker (20) have demonstrated that by far, greater numbers of the organism occur in the cream rising from a sample of milk than in the sediment or whole milk.

Studies on the presence of infection in market milk have not been so extensive, the dilution factor being responsible for the lower incidence of infection in such a product. Because Brucella organisms occur in milk and cream, their longevity in cream, butter and other milk products has been studied by several authorities. Samples of naturally infected cream show a large amount of infection because the fat globules carry up the organisms when they rise to the surface (21). Huddleson, in 1927, stated that the organism does not appear to multiply in milk and cream held at ice box temperature. He observed that the number of viable organisms decreases rapidly when held longer than eight days and after 60 to 90 days he was unable to recover the infection from the milk. Carpenter and Boak (21) observed the length of time the

organisms can live in cream and concluded that the percentage of butter fat in the cream and the hydrogen ion concentration of the skim milk in the cream are the controlling factors: "Evidently the higher the percentage of butter fat, the longer Brucella will live in a given sample of milk if stored at refrigerator temperature, because the more compactly the fat globules are packed, the less skim milk can the sample contain. The work of Sharp and McInerney (158), shows that the hydrogen ion concentration of a sample of cream or butter is approximately the same as that of the skim milk or buttermilk in it. This evidently indicates that butter made from sweet cream infected with Brucella abortus will still contain living abortus organisms and that if butter made from sour cream does not reach a pH of five, the infection may be carried over in this butter also.

The methods employed in manufacturing and processing cheeses, such as heating, souring and ripening, militate against the chance of these organisms being viable in cheeses, even though they may have been present in the various types of milk and cream from which they were made". From these findings, Carpenter and Boak concluded that Brucella abortus and Brucella melitensis are rare in cheese if not entirely absent, specially if they are examined 48 hours after their preparation.

Examination of Milk:

The following are the high points that are worth expounding and dwelling upon in tackling the problem of milk examination for the detection of Brucella:

1. Collection of specimens and their preservation before examination.
2. Isolation of Brucella from samples of milk.
3. Whey agglutination test.

1. Collection of specimens and their preservation before examination:

In collecting specimens of milk for bacteriological analysis extreme care must be taken to avoid the contamination of the samples with foreign bacteria and to check the growth of those already present.

Strict aseptic measures should be adopted while procuring the specimen in order to avoid any contamination with cocci, bacilli or other micro-organisms from the udder of the animal, from the hands of the milker and from the air as a result of undue long exposure of the sample. A number of ways are available to check the growth of the organisms already present in the milk. Among those most commonly employed are icing and other means of refrigeration.

These precautions are essential because milk samples may reach the laboratory in a condition that causes the death of the animals into which they are injected, or may undergo sufficient souring to destroy Brucella. Moreover, other bacteria may overgrow in plate media and cover Brucella colonies.

Gilbert, Coleman and Groesbeck (59) showed that 30 per cent glycerol will not destroy Brucella abortus in milk. Traum and Henry (173) used one per cent boric acid as a means of pre-

serving milk to be examined for Brucella abortus. Such preservation is sufficient to allow milk to be shipped considerable distances during the warm season without undergoing changes that would interfere with satisfactory laboratory examinations and inoculations into guinea pigs.

2. Isolation of Brucella from samples of milk:

In establishing the presence of the organism in milk, technical methods are important. Although Huddleson and his associates (86) demonstrated that the organisms were most numerous in gravity cream, other investigators obtained different results. Thus Fitch and Lubbehusen (51) considered that the sediment was the best inoculum, though they thought it safer to use whole milk as well. In 1933 Stockmayer (165) advocated the use of whole milk for the cultural isolation of Brucella. Karsten (97) found that fore, middle and end milk are equally good for cultivation, but emphasized that whole milk was the most efficient, centrifuged sediment comes next and cream the least suitable. For guinea pigs, he recommended a mixture of gravity cream and sediment.

Sheather has established the infectivity of certain samples of milk to guinea pigs indirectly by estimating the rise in the agglutinating power of the animals' sera following the injection of milk. He always found agglutinins within six weeks of injection. A guinea pig blood titer of 1:40 or higher may be taken as proof of infection. In 1932 Smith (160) demonstrated that an incubation period of four weeks in the

guinea pig was necessary for the appearance of agglutinins. In ten instances agglutinins were noted to disappear before the eighth week. He used the deposit from 25 ccs. of milk for each guinea pig and the tardy formation of agglutinins and their early disappearance may have been due to the very small numbers of Brucella inoculated. In 1922, Hagan (71), using high dilutions of culture, noted the appearance of agglutinins as early as the second week.

The reason why Brucella cannot be easily isolated from milk of high bacterial count may be that they are killed by the acids produced by saprophytic bacteria. When such milk is inoculated into guinea pigs, a specific agglutinin titer may occur, although Brucella may not be isolated from their tissues. It is probable that tissue reactions set up in the guinea pigs by inoculation of large numbers of saprophytes may cause premature death of small numbers of Brucella inoculated. Therefore, dirty milk gives a lower titer of agglutinins in guinea pigs than clean milk.

From these observations, we can conclude that most of the previous workers used animal inoculation for the detection of Brucella in milk. With the development of enriched media for cultural purposes, animal inoculation has been dispensed with, because it takes a long time to establish a definite diagnosis of the infection. On the other hand, culture media, in addition to the short time necessary for the detection of the infective organism, are easier to work with, cheaper, and the chances of getting a laboratory infection are much less.

3. Whey agglutination test:

In addition to the cultural methods and guinea pig inoculation, agglutination tests of the whey are of great value indicating a present or a past infection in the animal. When used as a routine procedure, they will be effective in detecting raw milk samples coming from badly infected herds. Bradley and his associates (11) examined 1788 samples of milk by means of the whey agglutination, and found out that 7.6 per cent of the positive reactors, discharged Brucella into the milk.

Giltner, Cooledge, and Huddleson (60) found that every case in which Brucella abortus was found by inoculating milk into guinea pigs, agglutinins were found in the milk, but Brucella abortus was not found in every case that gave positive test for milk serum. They concluded that either the bacilli were present in too small numbers to give lesions, or the presence of antibodies in the milk is a passive result from their presence in the blood of the animal. In 1932 Karsten (97) demonstrated that a milk-serum titer of 1:10 is regarded as positive, while a titer of 1:20 or higher is regarded as an index of presence of the organism. Cows that had aborted, due to this organism, became udder infected in 24 to 70 percent of cases. Elimination of the organism in the milk is quite constant. Infected cows that calved normally showed the organism in the milk in 25 per cent of cases. Moreover, he was able to show in certain instances that milk-infected animals might show a negative blood and whey titer.

In 1931 John and Pless (91) were able to show that of the

cows showing no blood reaction, 99.5 per cent gave no milk reaction, 96.7 per cent of the reactors in low dilutions (1:25 or 1:50) gave negative milk tests. It would appear, therefore, that if a blood serum agglutination test is negative or is positive with a serum dilution less than 1:100, the chances are small of finding Brucella in the milk. The higher the blood titer the greater the likelihood of the milk showing the organism. He concluded that the exact significance must depend upon the ability to isolate Brucella abortus from the milk.

Variations in the agglutinin titer of the milk serum during milking and the daily fluctuations were observed by Vellisto in Germany (175). He found out that the agglutinin titer changes during lactation. Increase of the agglutinins in the milk begins with the dry period and falls rapidly after calving. It is always lower than that of the blood serum. When blood serum titer was 1:320 and above, the milk was also positive, but when the titer was 1:40, no Brucella agglutinins were found normally in the milk.

Milk serum agglutinin content in some animals is therefore subject to many rapid changes. The antibodies may disappear completely in a few weeks, or a negative animal may become positive in the same time.

EXPERIMENTAL WORK OF PRESENT STUDY

INTRODUCTION:

In Syria and Lebanon, dairy products nearly top the list of our food commodities, and as these products afford a good medium for many pathogenic organisms, it seemed worthwhile to make a study of some of the organisms which are found in such products that threaten our national health and economy. The reported incidence of Malta fever in these two countries influenced the writer's choice of the subject.

Considering the reported endemicity of Brucellosis among animals and man in the Near East, it appeared worthwhile to study the incidence of the disease in Lebanon and Syria. It was also deemed of interest to establish as nearly as possible the source of the disease in selected representative areas and to demonstrate the Brucella species involved in human and animal infections.

MATERIALS AND METHODS:

In order to carry on the above mentioned project it was decided that a cultural study on the samples of milk brought to our laboratories would be made. It was also hoped that a study of the agglutinin titer of sera of individuals and animals in the infected areas could be carried out. Considering the importance of whey agglutination tests as an index in diagnosing past or present animal infection, such reactions were carried on every specimen of milk together with cultural studies, thus making available the opportunity of detecting any latent foci of this disease among healed

animals when no direct isolation of the organism from the milk was possible.

Though the source of the culture was known as to its being from a cow or a goat, yet the task of differentiation was approached in the same way as in dealing with an unknown organism. It is generally believed that B. melitensis is common to the goat, B. abortus to the cow, and B. suis to the hog. To a certain extent, this is true but overlapping might occur and the implication that each is confined always to a single host is absolutely without any basis.

Thus, the fact that a given strain comes from a cow, a hog or a goat is of value as supportive evidence in its identification and differentiation, but is never a conclusive proof for an absolute positive correlation.

The work was thus planned under the following headings:

1. Examination of milk samples: collected from different sources in Lebanon and Syria. This includes a thorough cultural study of strains isolated from such specimens, and the determination of the species involved in the infected area.
2. Whey agglutination tests: These were run on each sample of milk with each of the strains of Brucella to determine the type of infection, present or past, as manifested by the degree of agglutination with the specific antigen used.
3. Animal and human sera agglutination tests: These were run on human and animal sera using a pooled antigen of Brucella

melitensis, B. abortus and B. suis to determine the incidence of a present or past infection of Brucellosis among them.

1. Examination of Milk Samples: Collected From Different Sources In Lebanon and Syria.

Collection of Milk Samples:

The containers used to collect specimens were 20 ccs. vials, plugged with cotton-wool and sterilized in the dry heat sterilizer for two hours at 160-165°C. (Fig.1).

Fig.1. Vials used for the collection of milk samples.

The milkers' hands were washed thoroughly with soap and water and disinfected with 75 per cent alcohol; the animal's teats were wiped with 75 per cent alcohol.

The first streams of milk or strippings were discarded. The

The cotton plug was removed and the mouth of the vial flamed over an alcohol lamp and put directly under the udder. A pooled sample of milk was obtained by mixing portions of different specimens into a sterile vial, under aseptic conditions and stoppered with a sterile rubber stopper. The same procedure was routinely followed for every milk specimen in all instances.

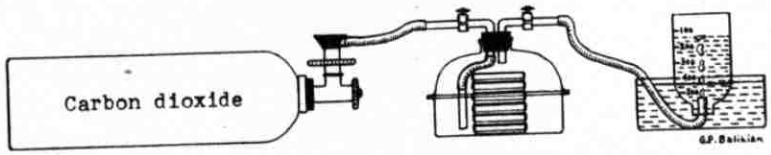
When samples were brought from distant places, extra precautions were taken depending upon the time that had to pass before they reached the laboratory. When time elapsed between milking and examination was less than ten hours the samples were put in an ice basin, however when the time needed was more than ten hours one per cent boric acid solution was added to the milk as recommended by Traum and Henry (173).

In most cases, however, the Beirut specimens were examined directly after reaching the laboratory, that is two to three hours was the maximum time elapsed between milking and examination, whereas the Damascus, Tripoli, and Mashghara, etc., specimens were examined a maximum of 24 hours after milking.

A heavily contaminated milk, containing a large flora of air bacteria presents a number of difficulties in the process of isolating the Brucella from the sample. As these organisms are not inhibited when found in great numbers by 1:700,000 crystal violet, they will overgrow the Brucella, and thus check their appearance; or they might change the pH of the medium to such a degree that Brucella, if present, will not be able to survive.

Culturing of Milk Samples:

Each vial was shaken properly to ensure good mixing, and a loopful of it was streaked on each of two Bacto-tryptose plates containing crystal violet to a final dilution of 1:700,000. The dye was to inhibit the growth of many gram positive organisms commonly found in milk. One of the Bacto-tryptose plates was incubated at 37° C. aerobically, while the other was incubated at 37° C. in the presence of 25 per cent carbon dioxide. (Fig. 2).



Apparatus for obtaining carbon dioxide and culture container for the isolation of Brucella abortus.

Fig. 2

The milk then was poured into 20 ccs sterile centrifuge tubes and centrifuged at 3,000 r.p.m. for 15 minutes. Three layers could be recognized:

1. a top layer containing the cream
2. a middle layer of casein in solution
3. the sediment.

A loopful from the cream layer was streaked on half of each of two Bacto-tryptose crystal violet plates. The remainder of the

cream was decanted into a disinfectant jar. The middle cream-free layer was decanted into a sterile 20 ccs vial and used later for whey agglutination. Similarly a loopful from the sediment was streaked on the remaining half of each of the two Bacto-tryptose crystal violet plates used above. One of the plates was incubated at 37° C. aerobically, while the other in the presence of 25 per cent carbon dioxide.

All four plates were incubated for a duration of one week, being examined daily for colonies of Brucella. When no such colonies appeared at the end of this period, the plates were left for another week in the incubator and discarded in case the results were negative. Suspicious colonies, however, when present, were easily distinguished from others by their light blue violet color on Bacto-tryptose crystal violet medium. Their size varied from one to four millimeters in diameter. (Figs. 3, 4, 5 and 6). The total number of colonies on a plate varied considerably depending upon the degree of udder infection. In my experiments, the number varied from 10-1600 colonies per cc of whole milk. Such colonies were fished and transferred to other Bacto-tryptose plates to obtain pure growth for further study. The culture was later studied from the standpoint of morphology, biochemical and agglutination reactions.

Choice of Media:

The following media were used for milk culture:

i. Bacto-tryptose medium

ii. Liver infusion medium

iii. Glucose inorganic salt medium.

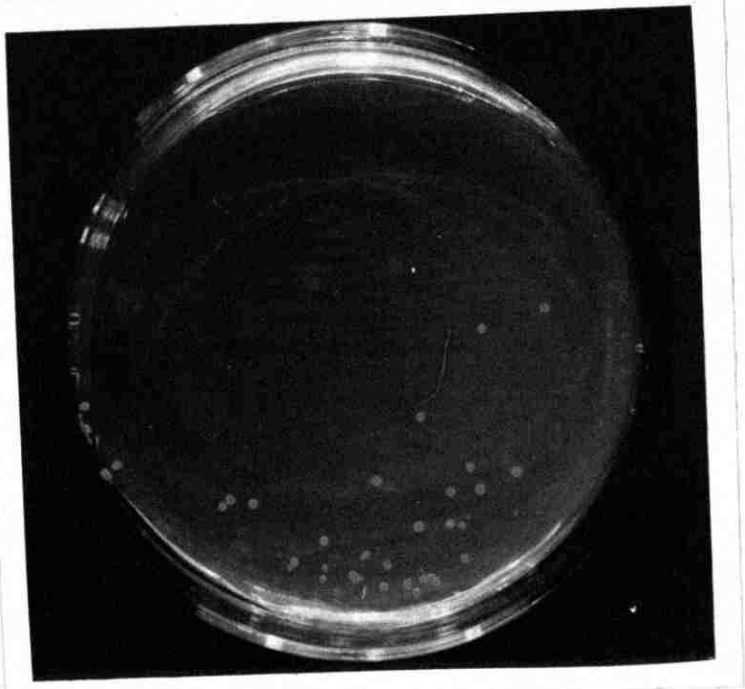


Fig. 3. Colonies of B. abortus isolated directly from milk

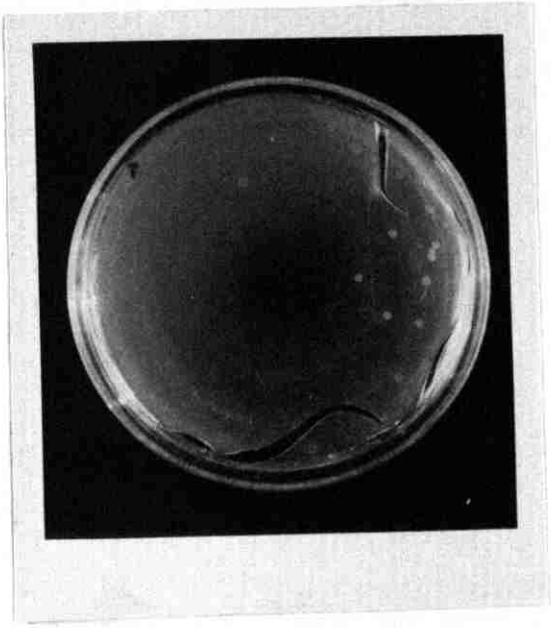


Fig. 4. Colonies of B. melitensis isolated directly from milk

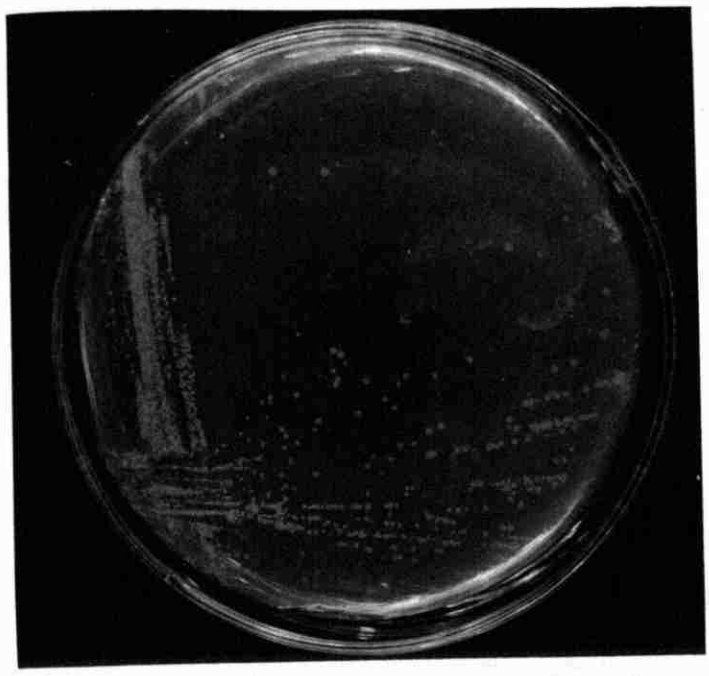


Fig. 5. A subculture of B. abortus

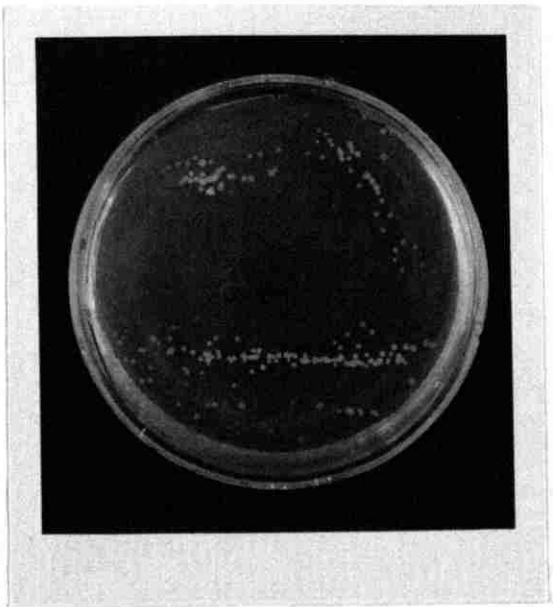


Fig. 6. A subculture of B. melitensis

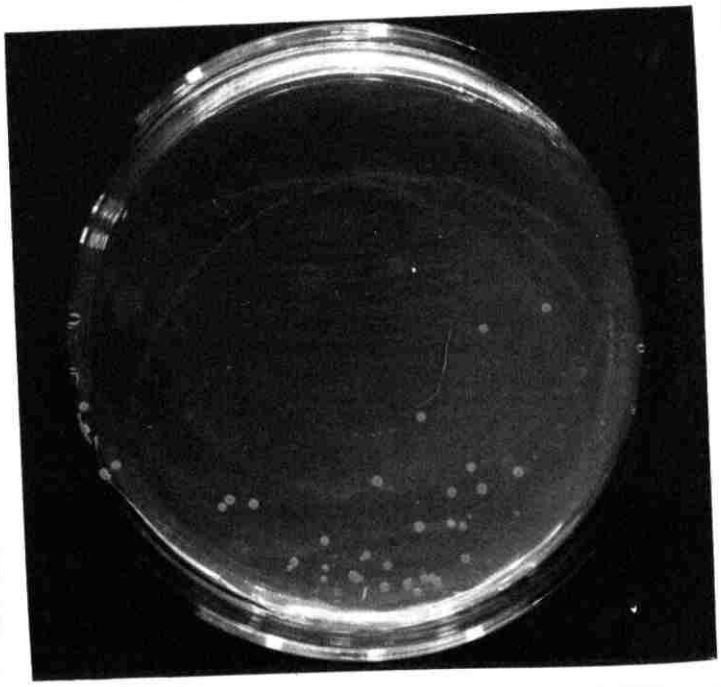


Fig. 3. Colonies of B. abortus isolated directly from milk

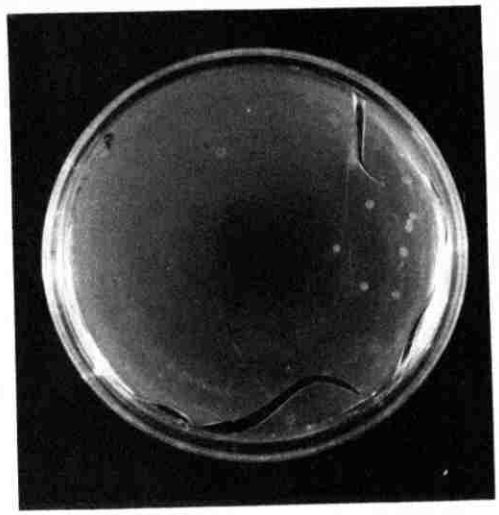


Fig. 4. Colonies of B. melitensis isolated directly from milk

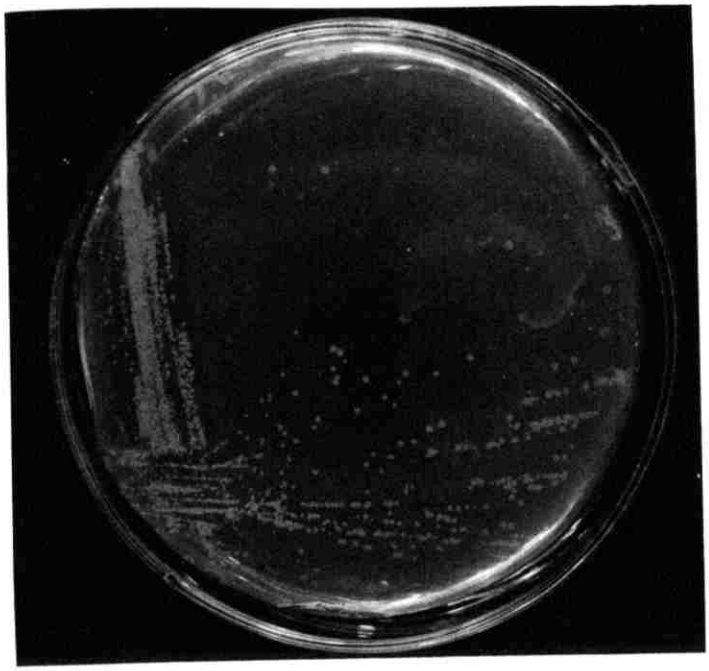


Fig. 5. A subculture of B. abortus

Fig. 6. A subculture of B. melitensis

The Bacto-tryptose agar medium proved to be the best. Its superiority lies in the fact that a quicker and richer initial growth was obtained and colonies were easily identified.

The addition of accessory growth factors such as thiamin, nicotinic acid amide, calcium pantothenate and biotin to Bacto-tryptose and liver infusion agar media showed no improvement in colony growth, neither in size nor in number. This may be attributed to the fact that these accessory growth factors were naturally found in the media in the required concentration for growth, thus the added amount was not utilized to a large extent.

Preparation of the Different Media:

Bacto-Tryptose Broth: Developed by the Difco Laboratories was prepared as follows:

Bacto-Tryptose.....	20 grams
Dextrose.....	1 gram
Sodium chloride.....	5 grams
Distilled water.....	1000 ccs

The mixture was heated for ten minutes to dissolve the ingredients. The pH was adjusted to 7.1. The mixture was then filtered through coarse filter paper and sterilized in the autoclave at 15 pounds pressure for 20 minutes. The final pH was dropped to 6.6 or 6.8.

Bacto-Tryptose Agar: Developed by the Difco Laboratories was prepared as follows:

Bacto-tryptose.....	20 grams
Dextrose.....	1 gram
Sodium chloride.....	5 grams
Agar.....	20 grams
Distilled water.....	1000 ccs

Before addition of the agar, the mixture was heated to dissolve the ingredients. The pH was adjusted to 7.1. It was then filtered through coarse filter paper. The agar was then added and the mixture sterilized in the autoclave at 15 pounds pressure for 20 minutes. The final pH was dropped to 6.6 or 6.8.

When the infective material was to be cultured for the presence of Brucella, a prepared 0.1 per cent aqueous solution of crystal violet was added to Bacto-tryptose agar, or broth in a sufficient amount to give the original dye a final dilution of 1:700,000.

Liver Infusion Agar: Described by Stafseth (164) was prepared as follows:

Bacto-liver ("Difco" Standardized ...	75 grams
peptone (Bacto).....	5 grams
Sodium chloride.....	5 grams
Agar.....	20 grams
Distilled water.....	1000 ccs

The Bacto-liver was added to 1000 ccs of distilled water, and infused at 50° C. for one hour. It was then heated to 80° C. and filtered. Peptone, sodium chloride and agar were added and the pH was adjusted to 7.2. The contents were decanted and placed in sterile flasks or tubes and sterilized in the autoclave at 15 pounds pressure for 30 minutes.

Liver Infusion Broth: Described by Stafseth (164) was prepared in the same manner, but with the omission of agar.

When infective material was to be cultured for the presence of Brucella, a prepared 0.1 per cent aqueous solution of gentian

violet was added to the infusion agar or broth in a sufficient amount to give the original dye a final dilution of 1:200,000.

For the Differentiation of the species of the genus Brucella the following dyes were used:

Thionin and basic fuchsin. These are certified dyes, made by the national Aniline and Chemical Company.

The dyes were prepared in a one percent stock solution in sterile distilled water. As these dyes are not soluble in water in this concentration, they were kept in well stoppered bottles for two days to ensure a complete solution, and to prevent evaporation. 0.1 per cent of the aqueous solution of thionin and basic fuchsin were added to the liver infusion and Bacto-tryptose agar in a sufficient amount to give the original dyes a final dilution of 1:30,000, 1:50,000 and 1:25,000 respectively. The dye suspensions were heated in flowing steam for 20 minutes, shaken well and, while still hot, were added to the melted medium before it had time to cool. This procedure resulted in a more uniform mixture of the dye suspension and a more uniform distribution of the dyes in the medium. The medium and dyes were thoroughly mixed and immediately poured into Petri plates. The plates were placed in a 37° C. incubator until the water of condensation disappeared.

Glucose inorganic salt medium: Described by McCullough and Dick was prepared as follows (122):

Ammonium sulphate.....	0.5 gram
Sodium chloride.....	7.5 gram
Dipotassium phosphate.....	1 gram

Magnesium sulphate.....	0.1 gram
Sodium thiosulphate.....	0.1 gram
Glucose.....	1 gram
Thiamin chloride.....	200 mgrams
Nicotinic acid.....	200 mgrams
Calcium panthothenate.....	40 mgrams
Biotin.....	1 mgram
Distilled water.....	1000 ccs

pH was adjusted to 6.8 - 7.2. The various ingredients were dissolved in distilled water and sterilized by filtration through Seitz filter or sterilized in the autoclave at 15 pounds pressure for 15 minutes.

2. Whey Agglutination Tests

Preparation of the whey:

The middle layer remaining after removal of the cream, was carefully transferred to 20 ccs sterile vials. The temperature of the milk was then raised to 37° C. in a water bath and ten drops of rennin solution were added to each vial followed by thorough mixing. The vials were then incubated at 37° C. for two to three hours in a slanting position (Fig. 7). Rennin coagulated the casein into a curd, which settled to one side and the clear whey was easily separated. Should any fat globules be still present, care was taken to remove them before the performance of the test for their interference in the agglutination. To do that, the whey was centrifuged at 3,000 r.p.m. for 15 minutes and left in the ice-box from 6 to 8 hours. The fat globules rise to the top. With a sterile Pasteur pipette about 0.5 cc from the middle portion

of the whey was removed without agitating the fat layer and the sediment. All precautions were taken to avoid the presence of even a small amount of fat or casein particle in the whey for those are bound to interfere with the agglutination reactions.

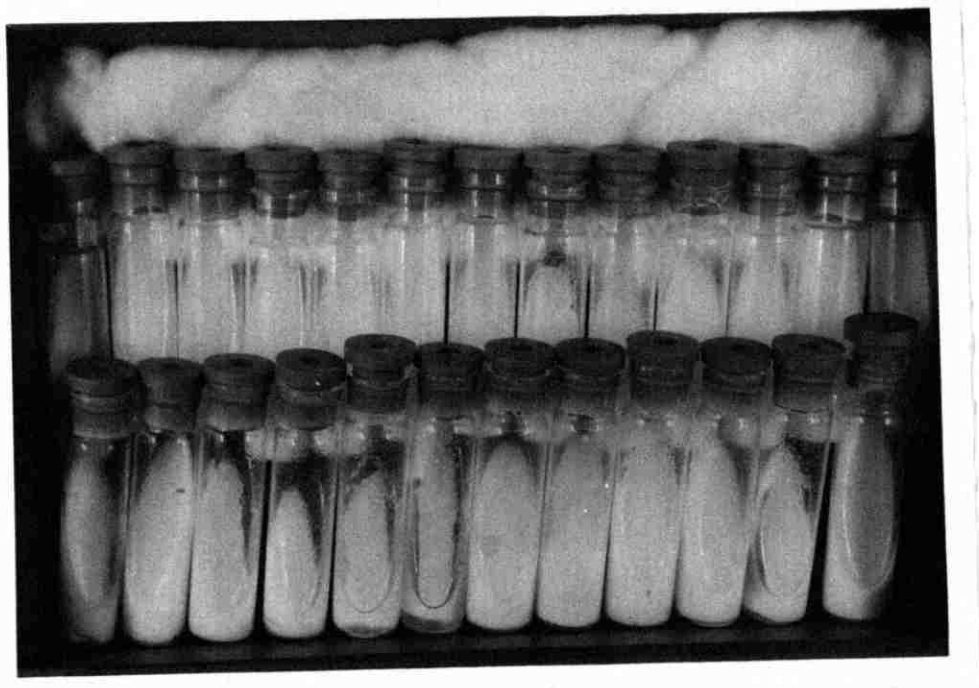


Fig. 7. Whey prepared for the agglutination tests

Preparation of the Antigen:

Smooth strains of B. abortus, B. melitensis and B. suis were used in the preparation of the antigen used.

Bacto-tryptose slants with 48 hours growth were washed each with 3 ccs of sterile physiological saline solution. To verify the absence of any contaminants smears were made from the saline suspensions and stained with Gram's method. Roux bottles containing 80 ccs of Bacto-tryptose agar were inoculated with pure cultures, using 2 ccs of bacterial saline suspension for each. The volume

of the suspension in each case was enough to cover the entire surface of the agar. The bottles were incubated for 48 hours at 37° C. and the growth washed off with 5 ccs sterile physiological saline. Smears stained with Gram's method were made from all washings to rule out the possibility of contamination. The suspensions were then transferred to sterile flasks using 10 ccs sterile pipettes and were heated at 56° C. for one hour. Phenol was then added to the heated suspensions to a concentration of 0.5 per cent. The flasks were then kept at room temperature in the dark and cultured daily to check sterility. Once sterile, they were stored as stock antigen at about 20° C. For test purposes, the stock antigen was filtered through several layers of sterile cotton to remove large clumps of bacteria and particles of culture medium. It was then diluted with sufficient 0.5 per cent phenolized physiological saline until the turbidity corresponded to 1½ billion organisms per cc. Fresh stock antigen batches were prepared every three weeks, for aged antigens failed to give positive results in a known positive serum.

Agglutination Tests:

The agglutination tests were carried out as in Widal test:

i. Eight small test tubes were arranged in a rack.

ii. 0.8 cc of physiological saline solution was placed in the first tube and 0.5 cc in each of the remaining ones.

iii. 0.2 cc of the clear whey was placed in the first tube. After the contents of this tube were mixed, 0.5 cc was transferred to the second tube and so on until the sixth tube was reached from which 0.5 cc was discarded. Thus a

serial dilution of 1:10 to 1:320 was obtained in the first six tubes.

iv. Tube No. 7 was the antigen control, containing 0.5 cc saline and 0.5 cc antigen.

v. A whey control tube No. 8 was made to contain 0.1 cc of whey and 0.9 cc of saline.

The tubes were incubated at 37°C. overnight and then allowed to cool off at room temperature for one to two hours, after which the first reading was made. Tubes were left at room temperature until the next day when the second and final reading was recorded.

A complete agglutination in any tube was characterized by a clear supernatant fluid and with agglutinated bacteria settling at the bottom of the tube.

Those test coming between complete and no clearing, were considered as partial agglutination.

3. Human and Animal Serum Agglutination tests:

Agglutination tests were performed on human and goat sera, following the same procedure as the one used for the Widal Test.

Human sera were obtained from all blood specimens coming into the Hospital Laboratories for routine work.

Goat sera were obtained weekly from Beirut slaughter house, during the period of December 1947 to April 1948.

PRESENTATION OF EXPERIMENTAL RESULTS.

Table I (A) presents the results of culture and whey agglutination tests on specimens of cow's milk from the Beirut area with their respective dates of collection, together with the percentages of positive cultures and agglutination tests. As an example of interpretation, on November 14, 1947, 28 cow's milk specimens were obtained from this area. Of these, two were found to produce positive B. melitensis cultures. Six of the 28 gave positive whey agglutination tests, three at an agglutinative titer of 1:10 and one each at titers of 1:20, 1:40 and 1:80 respectively. The remaining 22 specimens were negative. Interpretation of the other collection data in Table I (A) can be made along similar lines.

Table I (B) summarizes the results of similar experiments dealing with specimens of goat's milk collected from the Beirut area. Interpretation of this Table is identical with Table I (A).

Table I (C) deals with the experimental results of the agglutination tests performed on goat blood sera collected from goats in the Beirut area, giving the respective date of collection. The serum agglutination titers between 0 and 1:1280 with their respective percentages of the total are also given. As an example of interpretation, on November 22, 1947, 30 specimens were obtained from this area. Of these 13 were negative. None gave a positive agglutination titer of 1:20; one gave a titer of 1:40; two gave a titer of 1:80; four gave a titer of 1:160; two gave a titer of 1:320; one gave a titer of 1:640; and seven gave a titer of 1:1280.

TABLE I (A)

Beirut Area - Cow's Milk

Date	No. of specimens examined	Positive milk Culture. No.	Whey Agglutination Titers				
			Negative No.	1:10 No.	1:20 No.	1:40 No.	1:80 No.
6/11/47	24	0	22	2	0	0	0
14/11/47	28	2	22	3	1	1	1
20/11/47	24	0	17	3	4	0	0
11/12/47	25	0	18	1	2	2	2
19/12/47	29	0	27	1	0	1	0
28/12/47	23	0	21	0	2	0	0
3/1/48	25	0	18	1	2	2	2
10/1/48	28	0	22	3	1	1	1
15/1/48	53	0	44	4	4	1	0
23/1/48	47	0	43	2	2	0	0
TOTAL	306	2	254	20	18	8	6
PERCENTAGE		0.65	83.00	6.54	5.88	2.61	1.96

TABLE I (B)

Beirut Area - Goat's Milk

Date	No. of specimens examined	Positive Milk Culture. No.	Whey Agglutination Titers				
			Negative No.	1:10 No.	1:20 No.	1:40 No.	1:80 No.
28/8/47	46	0	34	0	6	4	2
4/9/47	46	0	40	0	4	2	0
29/9/47	25	0	21	2	2	0	0
16/9/47	25	0	21	2	2	0	0
2/10/47	25	0	21	2	0	2	0
20/10/47	30	0	26	2	0	1	1
2/11/47	28	0	26	1	0	1	0
13/11/47	25	0	20	2	3	0	0
20/11/47	25	0	23	0	2	0	0
2/12/47	30	1	24	4	2	0	0
15/12/47	30	0	24	2	2	1	1
21/12/47	25	0	20	0	2	2	1
8/1/48	28	1	23	2	2	0	1
21/1/48	25	0	21	0	2	1	1
3/2/48	30	0	26	1	1	2	0
18/2/48	32	0	29	2	1	0	0
26/2/48	25	0	22	1	1	1	0
12/3/48	30	0	30	0	0	0	0
TOTAL	530	2	451	23	32	17	7
PERCENTAGE		0.38	85.09	4.34	6.04	3.21	1.32

TABLE I (C)

Beirut Area - Goat's Sera

Date	No. of specimens examined	Serum Agglutination Titers							
		Negative No.	1:20 No.	1:40 No.	1:80 No.	1:160 No.	1:320 No.	1:640 No.	1:1280 No.
22/11/47	30	13	0	1	2	4	2	1	7
2/12/47	30	17	4	2	2	4	1	0	0
10/12/47	30	8	0	0	2	14	6	0	0
20/12/47	30	00	0	9	17	0	4	0	0
29/12/47	30	1	5	0	0	12	2	10	0
8/1/48	25	0	0	0	12	0	10	3	0
15/1/48	30	2	0	0	0	0	6	19	3
22/1/48	30	0	0	0	3	0	2	17	8
30/1/48	30	0	0	0	1	4	25	0	0
10/2/48	30	9	2	4	1	1	4	2	7
19/2/48	50	17	0	0	2	2	18	7	4
29/2/48	50	9	2	1	15	11	0	2	10
8/3/48	50	14	0	0	0	8	20	2	6
18/3/48	50	15	8	0	0	7	9	11	0
25/3/48	50	17	0	0	9	5	5	12	2
4/4/48	50	23	0	8	5	8	2	4	0
11/4/48	50	20	1	0	0	15	0	11	3
20/4/48	50	11	0	7	2	10	6	14	0
TOTAL	695	176	22	32	73	105	122	115	50
PERCENTAGE		25.32	3.17	4.60	10.50	15.11	17.55	16.55	7.19

The experimental results of agglutination tests on specimens of human blood sera collected in the Beirut area are shown in Table I (D). Interpretation is similar to data presented in Table I (C).

In Tables II, III, and IV are listed the specimens of goat's milk collected from the Tripoli, Laklouk and Damascus areas respectively. In Table V are listed the specimens of cow's milk collected from Mashghara area. Tables II, III, IV and V are arranged in a similar manner as Table I (A), and the interpretation is the same.

The schematic form presented in Table VI gives a summary of the data presented previously in Tables I (A), I (B), II, III, IV and V which concern all the milk specimen cultural studies. In other words, it summarizes in outline form the cultural studies showing the total specimens of milk obtained from different localities of Lebanon and Syria and the respective percentages of the positive findings. As an example of interpretation, a total of 1539 milk specimens were collected of which 14 Brucella strains were obtained, a percentage of 0.91.

Table VII differs from Table VIII in that it deals with the whey agglutination tests of all milk specimens obtained. In other respects, the two Tables are the same in their arrangement and interpretation.

Graph I is based on Tables I (A), I (B), I (C) and I (D) and deals with the agglutination titers of cow's milk, goat's milk, goat's sera and human sera in Beirut area. Titers of 1:10 and above are plotted against their percentage of positives.

TABLE I (D)

Beirut Area - Human Sera

Date	No. of specimens examined	Serum Agglutination Titers						
		Negative No.	1:20 No.	1:40 No.	1:80 No.	1:160 No.	1:320 No.	1:640 No.
11/10/47	24	22	1	0	0	1	0	0
13/10/47	27	24	1	1	1	0	0	0
18/10/47	30	27	0	3	0	0	0	0
19/10/47	25	25	0	0	0	0	0	0
21/10/47	28	27	0	1	0	0	0	0
31/10/47	25	23	0	2	0	0	0	0
1/11/47	25	24	1	0	0	0	0	0
3/11/47	25	25	0	0	0	0	0	0
5/11/47	25	22	2	1	0	0	0	0
10/11/47	25	25	0	0	0	0	0	0
23/11/47	25	19	2	3	1	0	0	0
24/11/47	30	23	0	5	2	0	0	0
27/11/47	30	28	0	0	1	0	0	1
28/11/47	25	22	0	0	0	2	1	0
1/12/47	27	20	1	4	2	0	0	0
4/12/47	30	25	0	0	2	0	0	3
7/12/47	30	20	4	0	0	5	1	0
9/12/47	25	15	5	0	4	0	1	0
11/12/47	25	19	2	3	0	0	0	1
18/12/47	25	19	0	2	0	3	1	0
7/1/48	25	20	0	5	0	0	0	0
11/1/48	30	25	2	2	0	1	0	0
20/1/48	25	16	0	6	2	0	0	1
28/1/48	30	26	0	2	0	0	0	2
TOTAL	641	541	21	40	15	12	4	8
PERCENTAGE		84.39	3.28	6.24	2.34	1.87	0.62	1.25

TABLE II

Tripoli Area - Goat's Milk

Date	No. of specimens examined	Positive Milk Culture. No.	Whey Agglutination Titers				
			Negative No.	1:10 No.	1:20 No.	1:40 No.	1:80 No.
20/10/47	24	0	21	2	0	0	1
15/11/47	28	1	26	1	0	1	0
12/11/47	30	1	14	5	6	3	2
TOTAL	82	2	61	8	6	4	3
PERCENTAGE		2.44	74.41	9.76	7.34	4.88	3.66

TABLE III

Laklouk Area - Goat's Milk

Date	No. of specimens examined	Positive Milk Culture No.	Whey Agglutination Titers				
			Negative No.	1:10 No.	1:20 No.	1:40 No.	1:80 No.
17/9/47	18	0	16	0	0	1	1
19/9/47	25	1	20	3	2	0	0
2/10/47	24	1	19	3	1	1	0
15/10/47	28	0	25	0	2	1	0
22/10/47	24	0	19	3	2	0	0
30/10/47	18	06	16	0	0	1	1
12/12/47	25	0	20	3	2	0	0
5/1/48	28	0	25	0	2	1	0
TOTAL	190	2	160	12	11	5	2
PERCENTAGE		1.05	84.21	6.32	5.79	2.63	1.05

TABLE IV

Damascus Area - Goat's Milk

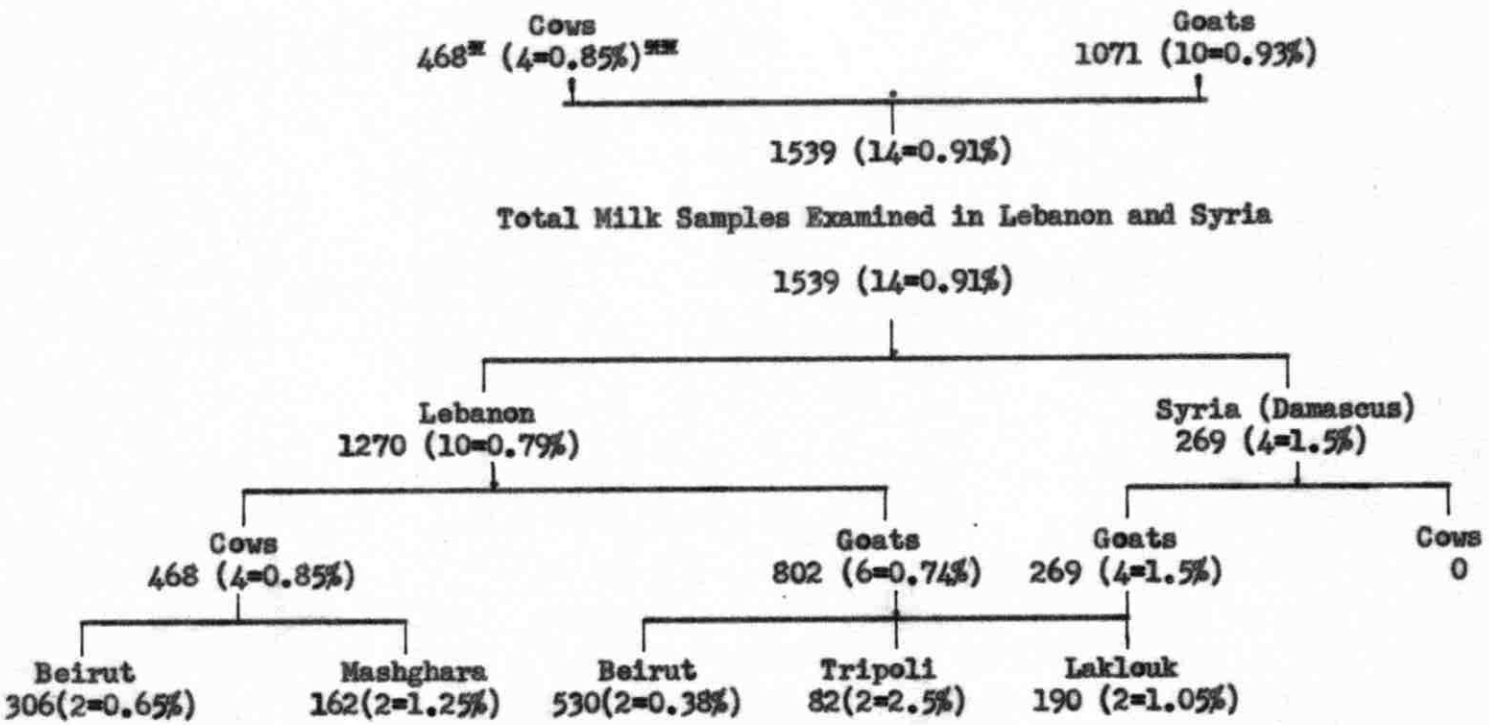
Date	No. of specimens examined	Positive Milk Culture No.	Whey Agglutination Titers				
			Negative No.	1:10 No.	1:20 No.	1:40 No.	1:80 No.
5/9/47	25	1	21	1	3	0	0
12/9/47	25	0	21	3	0	0	1
19/9/47	52	2	40	4	4	4	0
26/9/47	30	0	26	3	1	0	0
4/10/47	30	0	26	3	1	0	0
11/10/47	29	1	24	2	2	1	0
18/10/47	28	0	26	2	0	0	0
25/10/47	25	0	18	3	1	2	1
8/11/47	25	0	18	2	2	1	2
TOTAL	269	4	220	23	14	8	4
PERCENTAGE		1.48	81.78	8.55	5.20	2.97	1.48

TABLE V

Mashghara Area - Cow's Milk

Date	No. of specimens examined	Positive Milk Culture. No.	Whey Agglutination Titers				
			Negative No.	1:10 No.	1:20 No.	1:40 No.	1:80 No.
16/12/47	26	0	23	1	0	1	1
31/12/47	25	1	19	2	2	2	0
15/1/48	30	1	21	3	4	1	1
28/1/48	51	0	42	3	2	3	1
24/2/48	30	0	21	3	4	1	1
TOTAL	162	2	126	12	12	8	4
PERCENTAGE		1.23	77.77	7.41	7.41	4.94	2.46

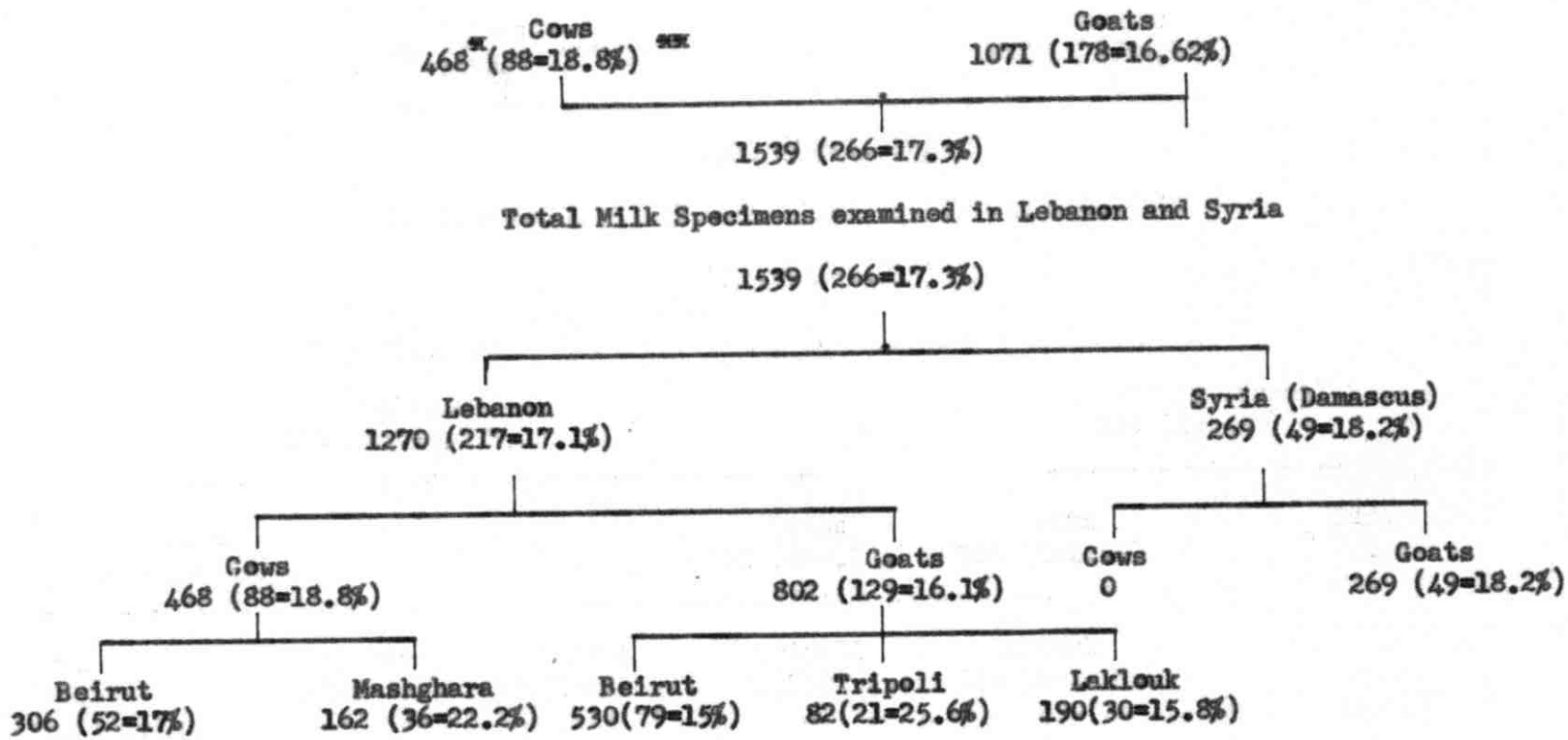
TABLE VI



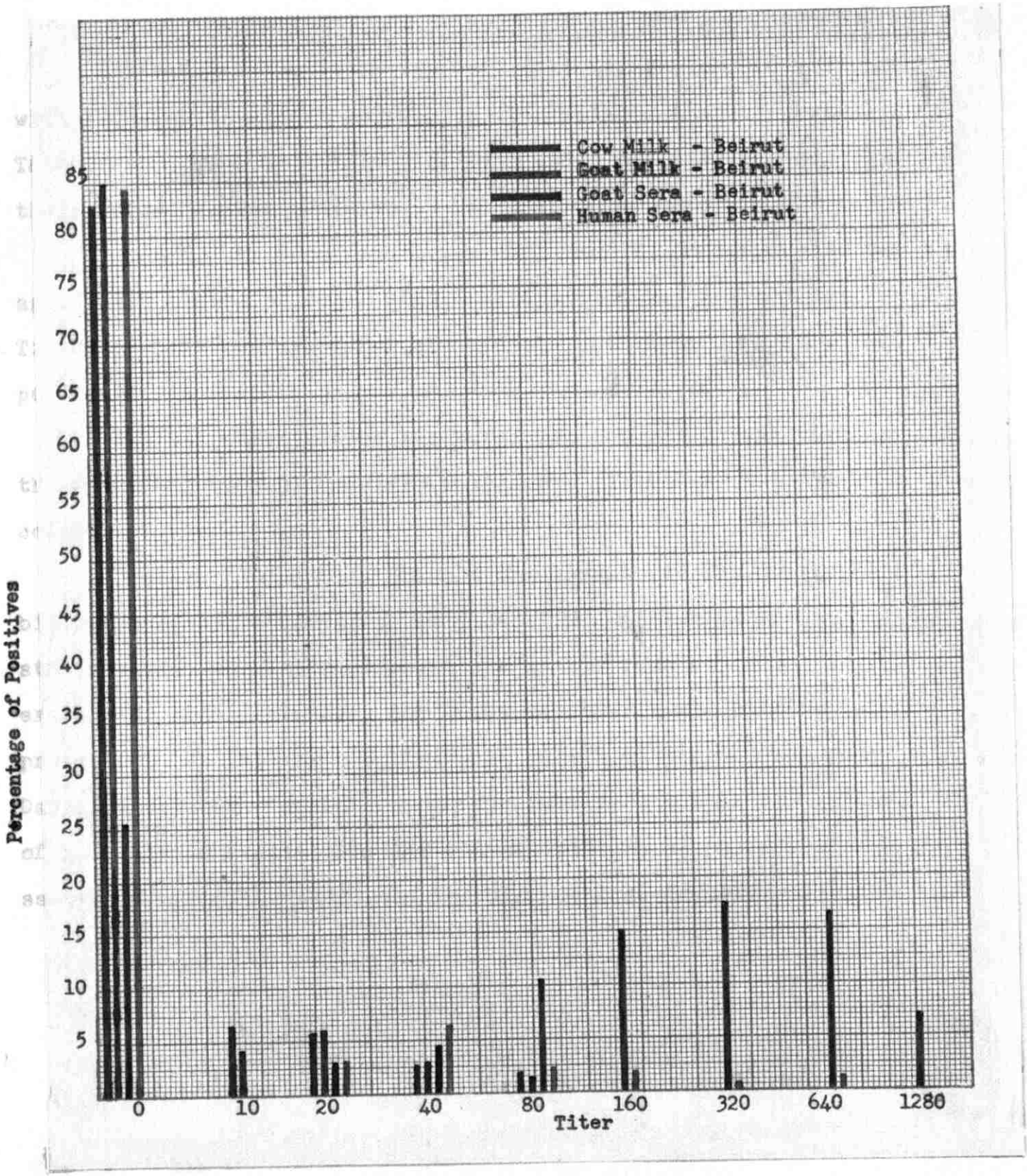
(*) Number of specimens obtained
(**) Number of positives obtained and their respective percentages

Strains isolated from goat's milk were all B. melitensis, while those isolated from cow's milk were all B. abortus.

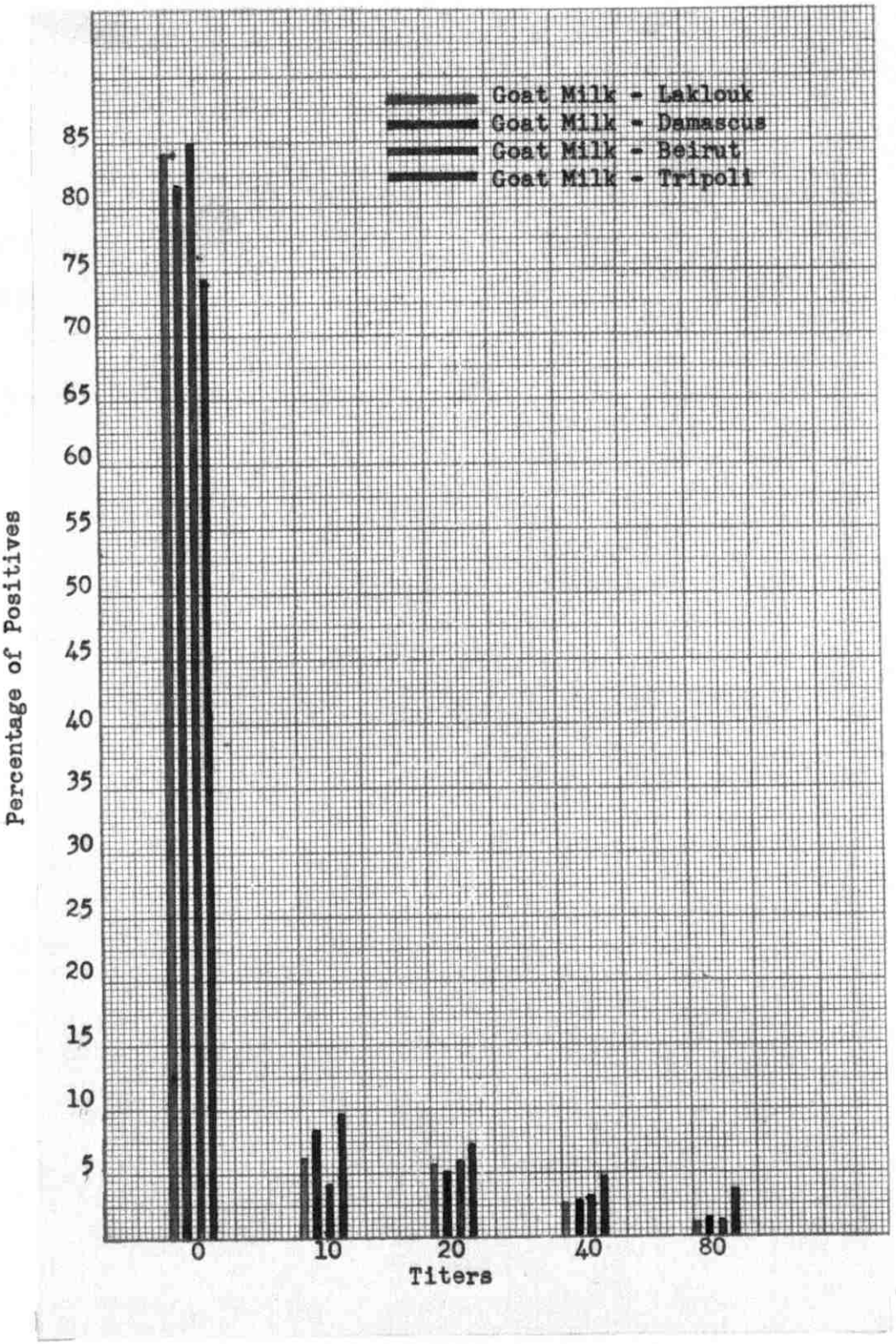
TABLE VII



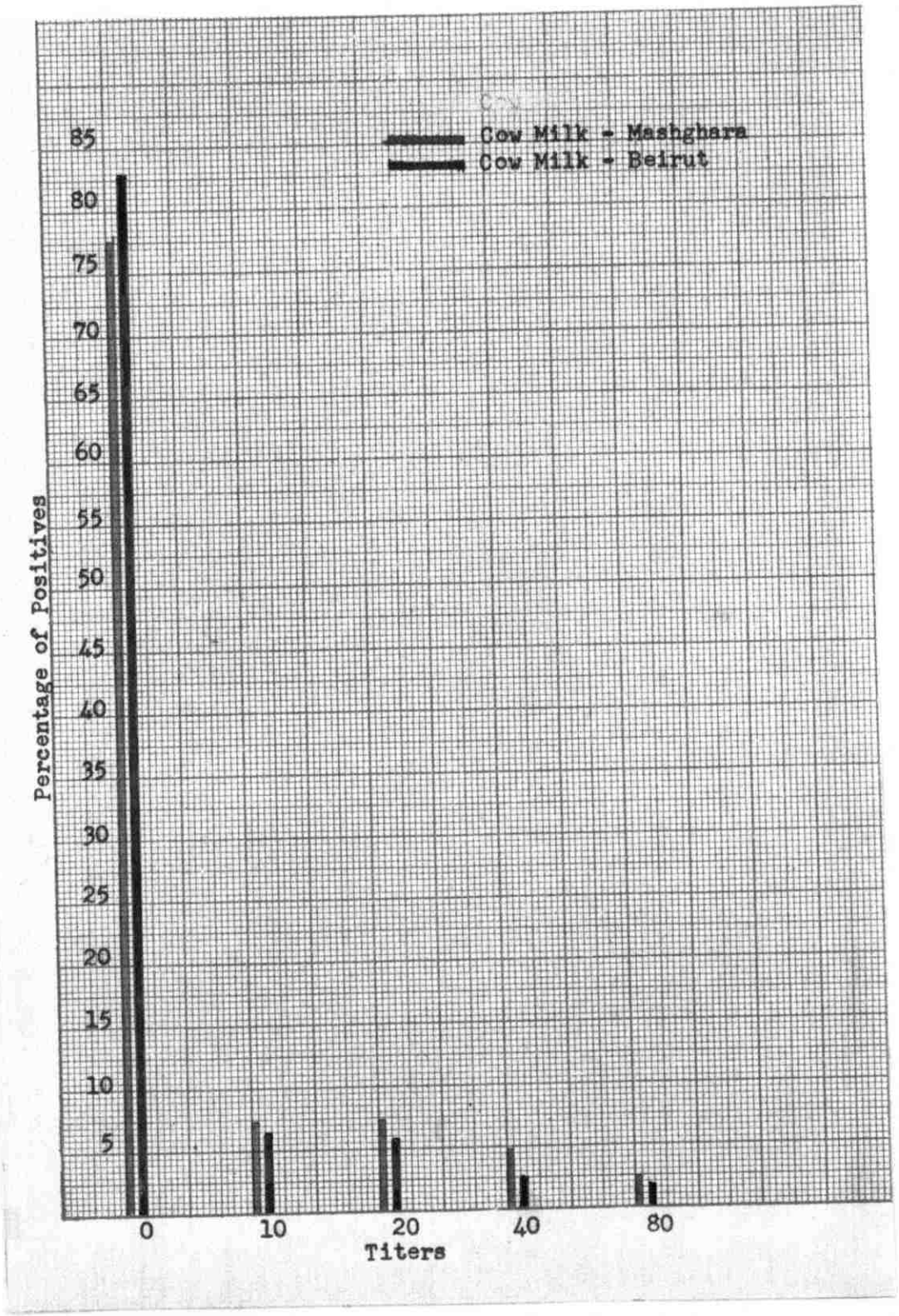
(*) Number of specimens obtained
(**) Number of positive agglutinations (showing a titer of 1:10 - 1:80), and their respective percentages.



Graph 1. Agglutinin titers of Cow Milk, Goat Milk, Goat Sera, and Human Sera.



Graph 2. Agglutinin titers of Goat Milk in Beirut, Laklouk, Tripoli, and Damascus areas.



Graph 3. Agglutinin titers of Cow Milk in Beirut and Mashghara areas.

TABLE VII

Cultural, Biochemical And Serological Studies of The Ten *Brucella Melitensis* Strains Isolated From Milk

Locality	Date	Source	No. of specimens examined	No. of colonies per cc. of milk	Colony characteristics on Bacto-Tryptose crystal violet	Growth in Bacto-Tryptose broth	Growth in gelatin	Growth in milk	Morphological characteristics	Nitrate-nitrite reduction tests	Indol production	H_2S formation	C O_2 requirement	Sugar Fermentation Tests: Glucose	Sugar Fermentation Tests: Sucrose	Sugar Fermentation Tests: Lactose	Sugar Fermentation Tests: Mannite	Growth in Dyes: Fuchsin 1:25,000	Growth in Dyes: Thionin 1:50,000	Growth in Dyes: Thionin 1:30,000	Serum Agglutination Titers: *Brucella abortus*	Serum Agglutination Titers: *Brucella melitensis*	Serum Agglutination Titers: *Brucella suis*	Slide Agglutination Tests	Motility
I. Damascus	5/9/47	Goat	25	30	Small, circular, flattened, amorphous, light blue-violet color, 3-4 mms. in diameter	Initial slight turbidity with surface growth. Later stringy tenacious sediment	No liquefaction. After 15 days of incubation dark, brownish, granular colonies	Slight alkalinity on 4th day	Gram(-) cocco-bacillary encapsulated	Positive	Negative	slightly positive on 3rd day	No CO_2 on primary isolation	slight acidity on 7th day	no change	no change	no change	+++ *	+++	+ **	80	640	40	Positive clumping with positive homologous sera	non-motile
II. Damascus	19/9/47	Goat	52	50	Small, circular, convex, amorphous, light blue-violet color, 1-2 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus, non-capsulated	Positive	Negative	Negative	No CO_2 on primary isolation	no change	no change	no change	no change	+++	+++	+	40	640	20	ditto	ditto
III. Damascus	19/9/47	Goat	52	160	Small, circular, convex, amorphous, light blue-violet color, 2-3 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus encapsulated	Positive	Negative	slightly positive on 4th day	No CO_2 on primary isolation	no change	no change	no change	no change	+++	+++	+	40	640	40	ditto	ditto
IV. Damascus	11/10/47	Goat	29	500	Small, circular, convex, amorphous, light blue-violet color, 1-2 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus, non-capsulated	Positive	Negative	Negative	No CO_2 on primary isolation	no change	no change	no change	no change	+++	+++	+	80	640	40	ditto	ditto
V. Laklouk	19/9/47	Goat	25	700	Small, circular, convex, amorphous, light blue-violet color, 1-2 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus encapsulated	Negative	Negative	Negative	No CO_2 on primary isolation	slight acidity on 7th day	no change	slight acidity on 7th day	slight acidity on 7th day	+++	+++	+	80	320	40	ditto	ditto
VI. Laklouk	2/10/47	Goat	24	1300	Small, circular, convex, amorphous, light blue-violet color, 2-3 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus encapsulated	Negative	Negative	Negative	No CO_2 on primary isolation	no change	no change	no change	no change	+++	+++	+	80	640	20	ditto	ditto
VII. Tripoli	15/11/47	Goat	28	60	Small, circular, convex, amorphous, light blue-violet color, 2-3 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus encapsulated	Positive	Negative	Negative	No CO_2 on primary isolation	slight acidity on 6th day	no change	no change	no change	+++	+++	+	20	640	20	ditto	ditto
VIII. Tripoli	12/12/47	Goat	30	90	Small, circular, convex, amorphous, light blue-violet color, 1-2 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus non-capsulated	Negative	Negative	Negative	No CO_2 on primary isolation	slight acidity on 7th day	no change	slight acidity on 7th day	no change	+++	+++	+	20	320	20	ditto	ditto
IX. Beirut	2/12/47	Goat	30	100	Small, circular, convex, amorphous, light blue-violet color, 1-3 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus non-capsulated	Negative	Negative	slightly positive on 4th day	No CO_2 on primary isolation	no change	no change	no change	no change	+++	+++	+	40	640	40	ditto	ditto
X. Beirut	8/1/48	Goat	28	70	Small, circular, convex, amorphous, light blue-violet color, 2-3 mms. in diameter	ditto	ditto	ditto	Gram(-) cocco-bacillus encapsulated	Positive	Negative	Negative	No CO_2 on primary isolation	no change	no change	no change	no change	+++	+++	+	40	640	20	ditto	ditto

* +++ = abundant growth
** + = slight growth

TABLE IX

Cultural, Biochemical & Serological Studies Of The 4 Brucella Abortus Strains Isolated From Milk

Locality	Date	Source	No. of specimens examined	No. of colonies per c.c. of milk	Colony characteristics on Bacto-Tryptose crystal violet	Growth in Bacto-Tryptose broth	Growth in gelatin	Growth in milk	Morphological characteristics	Nitrate-nitrite reduction tests	Indol production	H_2S formation	CO_2 requirement	Adaptation to aerobic conditions	Sugar Fermentation Tests				Growth in Dyes			Serum Agglutination Titers			Slide Agglutination Tests	Motility
															Glucose	Sucrose	Lactose	Mannite	Fuchsin 1:25000	Thionin 1:50000	Thionin 1:30000	B.abortus	B.melitensis	B. suis		
I. Beirut	14/11/47	Cow	28	200	Circular, flattened, amorphous, blue-violet in color, 2-4 mms. in diameter	Initial slight turbidity with surface growth. Later stringy tenacious sediment	No liquefaction	Slight alkalinity after 5 days of incubation	Gram (-) cocco-bacillus, rods more than cocci. Encapsulated	negative	negative	Moderate amount for two days	25% on primary isolation. No growth appeared with 15% carbon dioxide on subcultures	after 20 subcultures	slight acidity after 12th day	slight acidity after 12th day	slight acidity after 12th day	negative	+++ **	primary isolation+++ Later -	- *	1280	20	80	positive clumping with positive sera	non-motile
II. Beirut	14/11/47	Cow	28	1200	Circular, amorphous, flattened, blue-violet in color, 3-4 mms. in diameter	ditto	ditto	ditto	ditto	negative	negative	Moderate amount for three days	25% on primary isolation. Growth in 10% carbon dioxide on subcultures	after 15 subcultures	negative	negative	negative	negative	+++	ditto	-	1280	40	320	ditto	non-motile
III. Mashghara	31/12/47	Cow	25	170	Circular, amorphous, flattened, blue-violet in color, 3-4 mms. in diameter	ditto	ditto	ditto	ditto	negative	negative	Moderate amount for four days	ditto	after 21 subcultures	negative	negative	negative	negative	+++	ditto	-	1280	40	160	ditto	non-motile
IV. Mashghara	15/1/48	Cow	30	140	Circular, amorphous, flattened, blue-violet in color, 3-4 mms. in diameter	ditto	ditto	ditto	ditto	negative	negative	Moderate amount for four days	ditto	after 18 subcultures	negative	negative	negative	negative	+++	ditto	-	1280	80	320	ditto	non-motile

- * no growth

+++ ** abundant growth

DISCUSSION

TABLE VI - CULTURE STUDIES:

As previously mentioned, Tables I (A), I (B), II, III, IV, and V consist of the raw data upon which are based Tables VI, VII, and Graphs 1, 2, and 3. All discussion of results will, therefore, be confined to the summary tables and graphs.

By an examination of Table VI, it was found that in a survey of Brucella in milk in selected parts of Lebanon and Syria, 14 strains were isolated from examinations of a total of 1539 specimens. Ten of these were B. melitensis and four were B. abortus.

The ten B. melitensis strains were found in 1071 goat's milk specimens examined, which represents a ratio of 1:107, or 0.93 per cent. The four B. abortus strains were found from examination of 468 cow's milk specimens giving a ratio of 1:117 or 0.85 per cent indicating a roughly equal degree of infection with caprine and bovine strains.

There were 530 goat's milk and 306 cow's milk specimens collected from the Beirut area. From these four strains were isolated; two were found to be B. melitensis and two B. abortus or 0.38 per cent for B. melitensis and 0.65 per cent for B. abortus. This shows a higher ratio of active infection in cows than in goats, a ratio of 1.71:1.

A total of 190 goat's milk specimens were obtained from Laklouk, of which two were identified as B. melitensis giving an infection rate of 1.05 per cent or a ratio of 1:95.

In Tripoli, two B. melitensis strains were isolated from 82 goat's milk specimens, resulting in 2.5 per cent infection or a ratio of 1:41.

The Mashghara specimens, 162 in number, were all cow's milk and two B. abortus strains or 1.25 per cent were isolated from them, giving a ratio of 1:81.

The specimens obtained from Syria were all from Damascus. There were 269 goat's milk specimens, in which four B. melitensis strains were found, an infection rate of 1.5 per cent.

TABLE VII - WHEY AGGLUTINATION TEST:

A review of this Table reveals the following:

Out of 1539 specimens of goat's and cow's milk obtained from selected parts of Lebanon and Syria, 266 specimens gave a positive agglutination titer(*) making an incidence of 17.3 per cent.

In the 468 cow's milk samples from Lebanon and Syria examined, 88 specimens showed positive agglutinins, an incidence of 18.8 per cent, whereas 178 out of the 1071 goat's milk specimens examined were positive giving a percentage of 16.62. The ratio of the incidence of infection among cows as compared to that of goats is 1.13:1.

The total of cow's and goat's milk specimens obtained from Lebanon was 1270. 217 or 17.1 per cent of the total specimens gave a positive agglutinin titer. The percentage with cow's milk being 18.8 while that with goat's milk 16.1.

There were 530 goat's and 306 cow's milk specimens collected from the Beirut area. From these 79 or 15 per cent, and 52 or 17 per cent were positive respectively. The ratio of incidence of goat's as compared to cow's infection was 1:1.13.

The Mashghara specimens, 162 in number, were all cow's milk and 36 specimens or 22.2 per cent were positive.

(*) A titer of 1:10 to 1:80 was considered as a positive agglutination.

In Tripoli 21 or 25.6 per cent goat's milk specimens out of the 82 specimens examined were positive.

A total of 190 goat's milk specimens were obtained from Laklouk out of which 30 samples or 15.8 per cent were positive. The specimens from Syria were all obtained from Damascus. There were 269 goat's milk specimens in which 49 or 18.2 per cent were positive.

GRAPH I: AGGLUTININ TITERS OF COW'S MILK, GOAT'S MILK, GOAT'S SERA AND HUMAN SERA IN BEIRUT AREA.

A study of this graph reveals that the agglutinin titers of sera in general are higher than those obtained from milk. In majority of the agglutination tests, when goat's serum is used the titer has been 1:320, while with that of whey 1:20. This finding is in conformity with the concept which holds that antibodies are formed in the blood and pass thence into the milk (60). Both instances could be taken as an indication of a present or a past infection.

In comparing cow's and goat's milk of Beirut area it is seen that the majority of positive milk specimens from goats have a titer of 1:20 whereas those obtained from cows have a titer of 1:10.

The percentage of negative titers in cow's milk was 83, whereas that of goat's milk was 85. As a titer of 1:10 in milk is believed to be an indication of infection (97), 15 percent of the total specimens from goats could be considered as being obtained from infected sources. Judging from the same the cow's milk infection in the same locality was 17 per cent.

The percentage of negative titers in goat's sera was 25.32,

whereas that of human sera was 84.39. Considering a minimal titer of 1:80 as an indication of a present or past infection (77), the incidence of goat and human infections as judged from agglutinin titers in serum was 76 per cent and 6 per cent respectively (c.f. Tables I (C) and 1 (D)). Human infection is very low as compared with that of goats, as judged from the serum and milk titers.

This figure, however, does not present a fair estimation of true human infections, because the specimens examined were taken from the A.U.B. Hospital patients mostly from cities, thus excluding the villages where the percentage of infection is definitely higher due to lack of public health and sanitation.

The maximum number of positive human sera shows a titer of 1:40 which does not indicate an infection but could be due to cross agglutination resulting from other febrile illnesses or a very old infection.

From the above results it can be concluded that serum agglutinin titer is a better index in detecting a present or a past infection in animal than the agglutinin titer in the whey.

GRAPH 2: AGGLUTININ TITERS OF GOAT'S MILK IN BEIRUT, TRIPOLI, LAKLOUK AND DAMASCUS AREAS.

This graph shows that the goat's milk titers have a great deal of parallelism and uniformity in all localities.

The majority of positives in most localities have a titer of 1:10. The goat milk negative agglutinin titers of Tripoli, Damascus, Laklouk and Beirut were 74.4 per cent, 81.78 per cent, 84.2 per cent and 85 per cent respectively. Thus, Tripoli has the greatest per-

centage of infection (25.6 per cent), Damascus comes next (18.2 per cent), Laklouk ranks third (15.8 per cent) and the infection is least in Beirut (15 per cent).

These percentages obtained from milk titers are far less than the ones that would have been obtained from serum titers if the latter were tested, as is evidenced in the comparison of Beirut's goat's milk titers and serum titers of infected animals.

GRAPH 3: AGGLUTININ TITERS OF COW'S MILK IN BEIRUT AND MASHGHARA AREAS:

Cow's milk titers of Beirut and Mashghara run parallel to each other, the negative titers of which were 77.77 per cent and 83 per cent respectively. The incidence of infection in Mashghara is 22.2 per cent whereas that in Beirut is 17 per cent.

TABLES VIII AND IX: CULTURAL, BIOCHEMICAL AND SEROLOGICAL STUDIES OF THE B. MELITENSIS AND B. ABORTUS STRAINS ISOLATED FROM MILK:

A study of Tables VIII and IX reveals that all strains isolated from goat's milk belong to the species B. melitensis while those from cow's milk to the species B. abortus. There was much variation in the heaviness of infection among different specimens of milk obtained from goats and cows as detected by plate counts. A heavier flora of B. melitensis was observed on Damascus goat milk specimens and on those received from area of Laklouk as compared with the specimens obtained from Beirut and Tripoli. Furthermore, similar variations were observed among the milk specimens obtained

from cows. Beirut specimens showing a higher flora of B. abortus as compared with those of Mashghara specimens. Such results cannot be explained taking into consideration the lack of uniformity of time factor and variation in number of specimens received from one locality and the other.

In studying strains of B. melitensis isolated from goat's milk obtained from different localities, it was not possible to detect any major variations in colony characteristics. The colonies in all instances were small, circular convex or at times slightly flattened, amorphous light blue violet in color and varying from one to three mms. in diameter. The colony characteristics of B. abortus were the same as those of B. melitensis except for their larger sizes which ranged from two to four mms. in diameter. These findings agree closely with the previous reports describing the colonies of these two species (40). Furthermore, in fluid medium slight initial turbidity with surface growth and late stringy sediment formation with both species correspond closely with the classical description given for these organisms by Huddleson (84).

On gelatin medium B. melitensis showed dark, brownish and granular colonies after 15 days of incubation while the colonies of B. abortus although showed a granular appearance yet did not develop brownish pigmentation. None of the species liquefied gelatin. All the strains of B. melitensis and B. abortus showed a slight alkalinity in milk after four days of incubation.

Cocco-bacillary forms with B. melitensis strains were more common than the rod forms while the opposite was true for B.abortus. All the strains of B. melitensis and B. abortus with the exception

of strain II (Damascus), strain VIII (Tripoli), strain IX (Beirut), all belonging to the species melitensis, were encapsulated upon primary isolation. However, upon subsequent transfers on artificial media, with the exception of strain III of B. melitensis, the capsules were completely lost in a month time. This corresponds with the findings of Hershey, Huddleson and Pennel (14), Reiter (143) and Huddleson (83), where these workers report both capsulated and non-capsulated strains of B. melitensis and B. abortus in smooth newly isolated organisms.

Contradictory results were obtained in nitrate-nitrite reduction tests. The test was positive in six out of ten strains of B. melitensis and negative with all the strains of B. abortus. Nitrate-nitrite reducing power of the positive strains was completely lost after a month. Similar conflicting results were reported by Duncan and Whitby (34), Bergey (8) and Zobell and Meyer (187 VI). The former workers describing the whole group of B. melitensis as nitrate-nitrite non-reducers, while the latter workers as reducers.

None of the strains was able to attack tryptophane with the formation of indol. Slight variation was observed among different strains of B. melitensis as to their capacity in producing hydrogen sulphide. With some strains the results were completely negative while others showed a slight blackening on lead acetate paper after three to four days of incubation. With all strains of B. melitensis, Kligler's lead acetate medium showed no blackening. Furthermore, in a month's time hydrogen sulphide producing capacity of positive strains was completely lost. All the strains of B. abortus produced a moderate amount of hydrogen sulphide after few days of incubation

as tested by lead acetate paper. However, some time variation was observed among different members of the same species in respect to their hydrogen sulphide producing capacities. Strain I and II produced hydrogen sulphide for a duration of two days only. For strains III and IV the time was twice as long. All the strains of B. abortus were gradually adapted to grow under aerobic conditions in the absence of carbon dioxide. After such an adaptation strains I and II lost completely their hydrogen sulphide producing power while strains III and IV continued to produce hydrogen sulphide for a much shorter period of time. Such conflicting results have been reported by Kristensen (110), Thomsen (169) and Huddleson (81).

None of the strains of B. melitensis required carbon dioxide on primary isolation and no growth inhibition was observed in any of them in the presence of 25 per cent carbon dioxide. These findings do not agree with the reports of McAlpine and Slanetz (190) where they state that B. melitensis cultures are inhibited when inoculated in the presence of five to ten per cent carbon dioxide. All the strains of B. abortus required 25 per cent carbon dioxide upon primary isolation. No appreciable amount of growth was obtained when the amount of this gas was reduced down to 15 per cent in the culture medium. However, by gradual reduction of the amount of carbon dioxide within 15 to 25 subcultures, all the members of this species isolated from milk were adapted to grow in the complete absence of carbon dioxide (84).

Studying the sugar fermentation reactions, it was found that on the seventh day of growth glucose, lactose and mannite showed a slight degree of acidity with B. melitensis. There was no change in

sucrose with any of the strains of this species. Furthermore, within a month the fermentative capacities were completely lost. This last point can be explained by the following two alternatives: (1) either the organisms were not able to attack the sugars or (2) they did so, but the small amount of acid produced was rapidly neutralized by alkaline substances formed as a result of bacterial metabolism (123). B. abortus strains showed some variations in their fermentation reactions. Mannite was not fermented by any of the strains. Only strain I showed slight acidity after 12 days of incubation in glucose, sucrose and lactose. The rest of the strains did not ferment any of the sugars.

A concentration of 1:25,000 fuchsin was found not to inhibit any of the strains of B. melitensis. The growth of different strains of B. melitensis and B. abortus was also studied on thionin medium using a concentration of 1:30,000 as suggested by Mackie and Mc Cartney (115) and 1:50,000 as suggested by Jordan and Burrows (96). Appreciable growth was observed with B. melitensis on both of these plates, although the growth was much more profuse on the latter. Distinct colonies were also obtained with B. abortus when such strains were streaked on plates containing fuchsin in dilution of 1:25,000, and thionin in dilution of 1:50,000. However, no growth was observed when the dilution of the latter was brought down to 1:30,000. B. abortus strains adapted to grow under aerobic conditions in the absence of carbon dioxide were inhibited in the presence of 1:50,000 thionin. According to the results obtained, it can be safely stated that for differential purposes thionin in concentration of 1:30,000 can be used for freshly isolated organisms, while for

those organisms which have been subcultured for a prolonged period of time, a concentration of 1:50,000 thionin is much more suitable.

Although cross agglutination was observed when different strains of B. melitensis and B. abortus were used in the presence of an antiserum prepared against one of the species, yet the agglutination titer was always higher when a certain strain was brought in contact with its specific antiserum. Thus much higher titers were obtained when B. melitensis was used with its specific antiserum, than when B. abortus was used with the same. Such differences in agglutination titers follow closely the composition of the antigenic constitution of these species as reported by Wilson and Miles (177) stating that B. melitensis has a larger amount of "M" substance and a smaller amount of "A", while B. abortus and B. suis contain a larger amount of "A" and a smaller amount of "M". The ratio of "M" to "A" being 1:20.

While running slide agglutination tests similar results were obtained. Heavy clumping of organisms was observed when a species was brought in contact with its specific antiserum. Light clumping of organisms was the rule when a species was brought in contact with an antiserum prepared against a different strain in the same group.

SUMMARY AND CONCLUSION

1. A survey of goat's milk, cow's milk, goat's serum and human serum, obtained from selected representative areas of Lebanon and Syria was made for the presence of B. melitensis, B. abortus and their specific agglutinins. Strains of B. melitensis and B. abortus were isolated and their cultural characteristics investigated.

2. A total of 1539 milk specimens were examined of which 269 came from Syrian goats and 1270 came from either cows or goats of Lebanon.

3. The average incidence of infection among animals in Syria and Lebanon as judged from the number of positive findings of B. melitensis and B. abortus in goat's and cow's milk was 0.91 per cent. This reflects the relative incidence of active infection. The incidence of past infection among the animals as judged by the positive titer obtained from whey agglutination tests was 17.3 per cent. The current incidence of infection in cows and goats were of relatively similar magnitude, being 0.85 and 0.93 per cent respectively. Past infections as indicated by whey agglutination tests also were relatively equal for bovine and caprine strains, the incidences being 18.8 and 16.6 per cent respectively. Goat sera agglutination tests, on the other hand, indicated a much higher incidence of past infection among the goats to the extent of 67 per cent. Cow sera were not available for study but a survey of 641 human sera from the A.U.B. Hospitals gave an incidence of six per cent of positive agglutination tests for B. abortus. and B. melitensis

4. The average incidence of current infections for goats

from both Lebanon and Syria was 0.93 per cent. Infections were about twice as frequent in the Syrian goats (1.5 per cent) however, as in the Lebanese goats (0.74 per cent). Infection in Lebanese goats was higher in Tripoli (2.5 per cent) than at Laklouk (1.05 per cent) and least in Beirut (0.38 per cent). The incidence of current infections among Lebanese cows was 0.85 per cent but a higher rate of infection was found in Mashghara area (1.25 per cent) than in the Beirut area (0.65 per cent).

5. The average incidence of past infections for goats from both countries was 16.6 per cent. The incidence of past infections was 18.2 per cent for the Syrian goats and 16.1 per cent for the Lebanese goats. The incidence was higher in the Tripoli area (25.6 per cent) than in the Laklouk and Beirut areas (15.8 per cent) and (15 per cent) respectively. The incidence of past infections among Lebanese cows was 18.8 per cent. This figure was higher in Mashghara (22.2 per cent) than in Beirut area (17 per cent).

6. The cultural characteristics of the B. melitensis and B. abortus strains isolated from Lebanese and Syrian goats and cows, agreed essentially with the description given for these organisms by previous workers.

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