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Ontario Veterinary College
LABORATORY AIDS FOR THE DETECTION
OF BRUCELLOSIS IN CATTLE
UTILIZING MILK SAMPLES

A THESIS
PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF TORONTO UNIVERSITY FOR THE DEGREE OF
MASTER OF SCIENCE IN
AGRICULTURE

by
Sankar Ram Chandran
April 1948
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BIOGRAPHY

Sankar Ram Chandran was born at Ernakulam, Cochin State, a native state in India, on 17th August 1910. He received his primary and secondary education at Government High School, Ernakulam.

In 1931, he enrolled at the Bengal Veterinary College, Calcutta, India, from which he graduated in Veterinary Science in 1935. In the same year, he entered the Cochin State Government Service as a Veterinary Surgeon. In 1939, he was enrolled as a Post Graduate Student, at the Mysore Serum Institute, Bengalore, India. Until August, 1946, he worked as a Veterinary Surgeon for the Cochin Government. During this period he was fortunate in being able, without prejudice to his own duties, to serve for a short time with His Majesty's forces, inspecting and inoculating live-stock which were shipped from Cochin Harbour to various fighting fronts.

In October, 1946, he enrolled as a post graduate student at the School of Graduate Studies of Toronto University. His subjects, during a period of two years, covered Veterinary Science; Animal, Dairy and Poultry Husbandry.
ACKNOWLEDGEMENT

The author welcomes this opportunity to express his appreciation for the suggestions made by Dr. W. R. LeGrow through the course of his thesis. His grateful regards are due to Dr. A. L. MacNabb for offering him the facilities to carry out the work in the Ontario Veterinary College. Thanks are also due to Dr. C. A. V. Barker for his help in the collection of milk and blood samples, and to Dr. F. W. Schofield for his constant interest in the successful culmination of this thesis.

The author is ever grateful to Professors G. N. Ruhnke, R. G. Knox, and G. E. Raithby, of the Ontario Agricultural College, for the genuine interest they showed throughout the period of his training at the Veterinary College and the Agricultural College. The unreserved use of material from the Veterinary College is also greatly appreciated.
OBJECT OF THESIS

To determine the reliability of the Ring Test in the detection of agglutinins in milk of animals infected with Brucella abortus.

To find the correlation between the presence of agglutinins in the milk, as determined by the Ring test, and agglutinins in the blood as indicated by the blood serum agglutination test.

To ascertain the percentage of cows, showing a positive Ring test, which are actual shedders of Brucella abortus by way of the milk.

To ascertain also whether animals showing a negative Ring test may be emitting Brucella abortus organisms from the udder.
INTRODUCTION

Brucella abortus, the organism causing "Contagious Bovine Abortion", was first isolated by Bang, in 1897, from the foetus and foetal membranes of cows that had aborted. Bang's findings were later confirmed by many investigators. Since then, infectious abortion of cattle has become a problem of world wide importance. It is generally accepted that this disease is responsible for greater loss to the Dairy Industry than any other disease. Contagious abortion of cattle is widespread in the United States of America; and the economic loss resulting from this infectious disease has been estimated to exceed $50,000,000 per annum. From the point of view of both economy and good breeding, Bang's disease has become an obstacle to the progress of the cattle breeding industry. Workers in the field are persistently trying to bring this disease under control, and also to find an easier and more reliable means of diagnosis.

The usual methods adopted for the detection of brucellosis in cattle are blood and milk serum agglutination tests. Although the results of these tests are very encouraging when the tests are made by a competent laboratory worker, lack of agreement in the interpretation of the results limits their use.

Danish workers, Darnell (6), Seit (37) and Winther (47) have carried out extensive studies on the use of the Abortion Bang Ringprobe test, in the brucellosis programme in their
country. They have utilized this test, which was first reported by Hermann (22), in Germany, and have followed the technique as originally used, with the exception of filtration of the antigen. The initial antigen used in these studies was obtained through the courtesy of Dr. Jepsen of Denmark. The aforementioned workers claim that the Ringprobe test is reliable, sensitive, and easy to conduct.

Owing to the difficulty of obtaining translations, it was impossible to make a more definite detailed study of the work of these men. One of the objects of this thesis is to determine the reliability and sensitivity of the test.

Various investigators are of the opinion that milk agglutinins come from the individual infected quarters of the udder, and not from the blood stream. If this is true, the blood agglutination test cannot always be relied upon for detecting infected animals. In consideration of this possibility, the author also carried out work to determine the relationship between milk agglutinins and blood agglutinin.

Schroeder and Cotton (33), 1911, were the first to detect the presence of Brucella abortus in the milk of infected cows. Since then there have been conflicting reports by other workers as to the percentage of infected cows discharging the organism through their milk.

Authorities in Minnesota Medical School report that more than 80% of human brucellosis is caused by this bovine strain of Brucella abortus; and they claim the infection results from the
ingestion of raw milk (41). As infected milk proves a danger to public health, by transmitting "Undulant fever", an attempt is made in this experiment to find the percentage of infected cows that emit Brucella abortus organisms in their milk.
REVIEW OF LITERATURE

Presence of Brucella abortus in the Bovine Udder.

In 1911, Schroeder and Cotton (33), first isolated Brucella abortus from milk, while they were working in Mycobacterium tuberculosis. When they injected milk from tuberculous cows into guinea pigs, they found that certain lesions in guinea pigs resembled those produced by the tubercle bacilli. They were not able to recover the acid-fast organism, and concluded that the new bacillus was Brucella abortus.

In 1916, they reported that the uterus of cows harbours Brucella organisms only temporarily; and that, a few weeks after parturition, infection of the uterus clears up. They injected Brucella abortus into the nonpregnant bovine uterus, and found that the organisms disappeared in the course of a few days. Hence, they stated that the normal habitat of Brucella abortus was the udder of the cow. They succeeded also in producing the disease in guinea pigs, by feeding them milk from cows that were chronic carriers of the abortion bacillus.

The findings of Schroeder and Cotton were substantiated by Giltner and Bandeen (17). Smith (40) drew attention to the presence of Brucella abortus in the bovine udder.

Mitchell and Duthie (28), 1920, have confirmed these findings, and have shown that even in oestrus the infected animal does not harbour the organism in the uterus. They also claimed that, in a large proportion of infected animals, Brucella abortus is harboured in the udder.
Mohler and Traum (30), were able to isolate Brucella abortus from lesions in liver, spleen, and testicles of guinea pigs, which had been inoculated with materials from tonsils of milk consuming children.

Fabyan (9), claimed that milk furnishes a medium in which Brucella organisms may multiply over long periods of time, and that it remains as a constant source of infection for other cows.

Zwich and Krage (48), demonstrated the presence of Brucella organisms in milk, by cultural methods. They were able to demonstrate the presence of these organisms in the milk of goats within twenty-four hours after intravenous injection of the abortus bacillus.

Evans (8), succeeded in isolating from milk organisms which she believed to be Bact. abortus.

Cooledge (4), in 1916, demonstrated that if a pure culture of Bang's bacillus were introduced into the udder of a cow, it caused the appearance of agglutinins in the milk.

Stafseth (42), 1920, conducted experiments to find out whether Bang's bacillus was harboured in the deeper layers of the mucous membrane of the non-gravid uterus, and concluded that the bacillus does not persist indefinitely in the genital tract and does not penetrate the layers of the mucous membrane there.

Huddleston (25), in 1920, was able to isolate Brucella abortus from milk.
Tweed (44), 1923, found that the fore milk from the udders of infected cows was more likely to produce typical lesions of Brucella abortus in guinea pigs than the strippings. This would seem to indicate that the organisms are more prevalent in the fore milk than in the strippings.

Gilman (13), 1930, studied milk from all four quarters of 34 cattle. As a result he tentatively assumed that quarters showing agglutinins of 1:80, or more, are infected with Bang's bacillus and may eliminate the organism at any time; and that quarters, showing agglutinins lower than 1:80, only in rare instances contain or eliminate the organism.

Gilman and Milks (15), have been able to isolate and type 113 bovine strains, from 117 strains of Brucella recovered from milk in New York State.

Gill (12), after inoculating Brucella abortus in the mammary vein, succeeded in isolating the organism from all four quarters of the udder.

Hayes and Barger (19), 1935, were able to isolate Brucella several times from the udders of cows.

Henry, Haring and Traum (20), 1935, stated that cows with a blood agglutination titre of 1:100, or higher, are actual or potential shedders of Brucella abortus in milk; and that cows with a titre of 1:50, or lower, may shed the organism at times.

In 1936-37, Fitch and Bishop (11), were able to isolate Brucella abortus from milk samples with agglutinins in a dilution of 1:25, as well as from samples showing no reaction in this
dilution. They inoculated guinea pigs, and demonstrated the presence of *Brucella abortus* in samples which had agglutinins only in 1:5 dilution.

All the investigators quoted above were unanimous in their opinion that the udder is a habitat of *Brucella abortus*.

**PERCENTAGE OF INFECTED COWS ELIMINATING *BRUCELLA ABORTUS* IN THEIR MILK**

Since the discovery of Bang's bacillus in milk, there have been conflicting reports as to the percentage of infected cows that eliminate the organism through this channel.

Cotton (5), 1913, found, of 19 cows having records of abortion, 18 cows or 95 per cent, were eliminating *Brucella* in their udder secretion.

In 1919, Sedden (36), reported that 71 per cent of cows reacting to the agglutination test emitted Bang's bacillus through the udder.

Schroeder and Cotton (34), in 1924, showed that 83 per cent of infected cows, having a titre of 1:200, or higher, produced abortion bacillus in the milk.

Sheather (38), found 34 per cent of positive animals discharged *Brucella abortus* in their milk.

In 1924, Fitch and Lubbehusen (10) observed that 29.1 per cent of infected cows shed abortion bacillus through the udder.

Carpenter (2), 1926, reported that 66 per cent of cows
showing Bang's disease, were capable of eliminating the organism from their udders.

King and Caldwell (26), 1929, stated that 41 per cent of cows whose titre was 1:200 or more, eliminated Brucella organisms in their milk.

Mitchell and Humphreys (29), in 1931, found that 75 per cent of cows excreted Bacillus abortus with the udder secretion.

In 1931, Gilman (14) stated that Brucella abortus was found in the milk from 63 per cent of cows having a titre of 1:80, or higher.

Hayes and Barger (19), 1935, found that 77.5 per cent of infected cows were shedding Bang's organism through the channel of the udder. They also observed in the course of their study, that over 21 per cent of the cows shed the organism in milk, either before agglutinins appeared in the blood, or at a time when the blood agglutinins had receded to negative, or to positive at a dilution of only 1:50.

Henry, Haring and Traum (20), in 1935, found that 96.3 per cent of the udders they examined were discharging Brucella abortus, when the corresponding blood titre was 1:200, or higher.

Proscholdt (31), stated only 3 per cent of cows, with blood serum titre of less than 1:100, were capable of eliminating Bang's organism through the udder; but that cows with whey titres of 1:80, or higher, almost certainly were shedders.
Viriden (45) observed that a low blood titre was indicative of udder infection. He found that 50 per cent of the animals studied, with blood titre of 1:50, showed the abortus organism in the udder. He found that, also, 56 per cent of cows with blood titre of 1:70 were capable of eliminating Brucella abortus in their udder secretion.

Gwatkin (18) found 52 shedders among 102 cows which showed blood serum titre of 1:100, or greater. He also found a "vaccinal strain" of Brucella abortus in milk, when the blood serum was negative to agglutination tests.

The above reports of the various investigations indicate that a considerable number of cows infected with Bang's disease are capable of eliminating Brucella abortus in their milk.

THE RELATION BETWEEN MILK AGGLUTININS AND BLOOD AGGLUTININS

In 1916, Cooledge (3) stated that active infection of the udder with Brucella abortus produces antibodies in milk, and that these antibodies are not due to diffusion of antibodies from the blood. To emphasize this, he pointed out that milk with a negative or low titre has been taken from cows with a high blood titre; and that milk may have high agglutinins when the blood has low agglutins. He stated also that when the antibodies for Brucella abortus are present in high concentration in the milk, the organism is usually found; and that when the organism is not found accompanying the antibodies, it is only because the infection is disappearing, or is so slight that there are too few organisms present, in the 5 cc. of
milk used for inoculation, to cause lesions in guinea pigs.

In 1923, Smith, Orcutt and Little (39) working on udder infection, concluded that milk agglutinins come mainly from the udder and not from the blood.

Tweed (44) in 1923, observed that Brucella agglutinins of milk vary among individual quarters of the same udder, which indicates that they are of local origin. He stated that antibodies may be present in milk when there is no active infection of the milk: this was proved when 5 cc. of whole milk were injected into guinea pigs.

In 1931, Gilman (14) stated, "It seems certain that udder agglutinins do not come from the blood stream, but are produced locally in each quarter of the udder."

It would seem from these reports, that the milk agglutinins originate from the udder itself, in most cases, and are not transferred from the blood stream.
EXPERIMENT

Materials used for Problem

Milk and blood were collected from 15 herds in the Guelph area. Guinea pigs were furnished by the Ontario Veterinary College. A total of 320 cows and 124 guinea pigs were included. The cultures of Brucella used in the experiment were from a smooth strain stock culture, obtained from the United States Bureau of Animal Industry.

PROCEDURE

COLLECTION OF MILK AND BLOOD SAMPLES

At each herd visited, blood samples were drawn first. The cow was controlled in a stanchion with a halter, and the jugular vein raised with a choke rope. The area on the jugular vein, where the needle was inserted, was cleaned with a 60 per cent alcohol solution. A sterilized dry test tube of $\frac{3}{8}$" x 4" size, with needle attached, was held securely in the hand; the needle was quickly inserted into the vein; and about 5 to 10 cc. of blood were withdrawn. The choke rope was immediately released, and the needle removed from the vein. The test tube was then stoppered, labelled, and numbered. In addition to this, a special sheet for the identification of each sample collected was also prepared. Separate sterilized needles and receptacles were used for each animal. The samples were kept in a cool place.
Milk samples for the tests were collected at the same time. Because Tweed (44) has stated that the foremilk contains more Brucella organisms than the strippings, foremilk was collected for this experiment. The udders of the cows, from which milk samples were taken, were washed with Roccal solution (1 tablespoonful to a gallon of warm water). Individual udder cloths were used for each animal, and the udders were wiped dry. The teats were cleaned with cotton, soaked in 60 per cent alcohol solution.

About two ounces of milk from each quarter of the udder were expressed into each of four separate clean, dry, sterilized milk sample bottles. The bottles were stoppered and labelled. Either the name or tag number of the cow, and the designation of the quarter, was noted on each bottle by using signs, R.F., R.H., L.F., and L.H. (Right front, Right Hind, Left front and Left Hind).

Blood and milk samples thus collected were brought to the Research Laboratory of the Ontario Veterinary College, and the tests were conducted as soon as possible. If many tests were to be made, it was done on subsequent days. Samples were kept in the refrigerator overnight. Guinea pigs were inoculated with these milk samples.

**THE ABORTION BANG RINGPROBE TEST**

This test has been designated as the "Ring Test". The antigen used for the test was hematoxylin stained antigen. The procedure of making hematoxylin stained antigen for this experiment was the one adopted by Jepsen, of Denmark, with modification by LeGrow; this was checked several times with Jepsen's antigen
before being used. The results are given in Table 1.

One gram of hematoxylin purissimum was dissolved in 25 cc. of absolute alcohol. The solution was heated in a water bath, to 50 C. When the stain was completely dissolved, it was removed from the water bath; and the volume was increased to 100 cc., with the addition of distilled water. This was solution No. 1. Nine grams of ammonium aluminum sulphate were dissolved in 100 cc. of distilled water in a separate flask. When they were dissolved completely, 30 cc. of glycerine were added, to make the volume 130 cc. This was solution No. 11.

Solutions 1 and 11 were mixed. To the mixture were added 0.17 grams of potassium iodide, dissolved in 2 cc. of distilled water. After 15 minutes, the mixture was diluted to 5 times its original volume by the addition of a 10 per cent solution of ammonium-aluminum sulphate. The mixture was allowed to stand for 20 hours. At the end of this period, it was filtered through filter paper into another clean dry flask; and was stored in the refrigerator at 40 F. This mixture constituted the hematoxylin stain.

Beef liver infusion agar, Bacto tryptose agar, and potato infusion agar have been used successfully in Laboratories to isolate and grow Brucella abortus. Of these, potato infusion agar medium was the one used in this experiment. This medium has proved quite satisfactory for growing Brucella abortus. Sound raw potatoes were washed in water. They were pared and sliced into thin pieces, with minimum exposure to air. Two hundred and fifty grams of potatoes were placed in 1000 cc. of distilled water in a flask. The flask was stoppered and the mixture held overnight in a water bath at 60 C.
The next morning the mixture was filtered through filter paper, and the volume again brought up to 1000 cc. The following ingredients were weighed separately, and added to the potato infusion:

- Sodium chloride U.S.P. 5 grams
- Bacto-peptone 10 grams
- Beef Extract 5 grams
- Dextrose U.S.P. 10 grams
- Agar U.S.P. 30 grams

The flask was rotated several times. The mixture was then heated in flowing steam until the ingredients were complete dissolved.

The medium was adjusted to a pH of 7.4. After final autoclaving, the pH was approximately 6.8, which is the most suitable pH for the growth of the organism.

When the medium was dissolved, it was poured into sterilized, flat-sided 'Roux bottles', and stoppered. A quantity, sufficient to make a quarter-inch layer when the bottle was placed in a horizontal position, was added to each flask. The final sterilization was effected by autoclaving at 15 lbs. pressure, for 20 minutes. At the end of the sterilization period, the medium was removed from the autoclave; and the bottles were placed in a horizontal position while the medium solidified. The medium thus prepared was stored in the refrigerator until used.

Typical strains of Brucella abortus should be selected and cultivated for the antigen. The strain used in this experiment
was a United States Bureau of Animal Industry strain, No. 119. This strain of Brucella was sub-cultured on tryptose agar media slants. Tryptose agar medium was prepared by dissolving 41 grams of Bacto-tryptose agar in 100 cc. of distilled water. The mixture was heated over flowing steam. When completely dissolved, it was removed from the steam; and the pH was adjusted to 7.4. Six to eight cc. of the mixture were poured into each of the several test tubes. The tubes were stoppered with cotton wool, and sterilized by autoclaving at 15 lbs. pressure for 20 minutes. Then they were removed from the autoclave, and placed in a slanting position to solidify. The slants were stored in the refrigerator.

Tryptose agar slants for seed cultures were inoculated with growth from the stock culture. The mouth of the tube was flamed, while it was opened and closed. The cultures thus made were incubated for 24 to 48 hours, at 37° C. The growth on these tubes was washed off with sterilized physiological saline. Each tube was examined microscopically for contaminants. The washed growth was then pooled in another tube containing sterilized saline, to make a density of approximately tube No. 10 McFarland nephelometer. This constituted the seeding suspension.

Sufficient seed suspension was added to each bottle of culture medium, to permit uniform distribution over its surface. The flasks were then placed in an incubator at 37° C. The bottles were inverted, to prevent the water of condensation from coming into contact with the surface of the agar. The growth was examined daily, and contaminated bottles were removed.
When maximum growth was obtained, in from three to five days' time, the bottles were removed from the incubator. The water of condensation was removed without contacting the bacterial growth. The bacteria from the Roux bottles were washed off in a minimum amount of physiological saline solution. The suspended organisms were pooled, filtered through several layers of sterile cotton wool, and collected in a flask. Then the contents of the flask were centrifuged for one hour, at 2500 revolutions per minute. At the end of this period, the bacteria were deposited at the bottom, leaving the saline fluid on the top. This fluid was siphoned off. The bacterial deposit was re-suspended in physiological saline, and washed by renewed centrifugalization three times.

After the third washing, the bacteria were suspended in the hematoxylin stain. The mixture was heated to 65° C. for five minutes in a water bath, and rapidly cooled. The stained organisms were centrifuged again, for one hour, and the supernatant fluid stain was removed. The stained Brucella organisms were washed three times with acidified distilled water, pH 4, and centrifuged after each washing. The stained and washed Brucella abortus was then suspended in phenolized physiological saline. The final volume was increased to 15 times the volume of the bacterial deposit, by the addition of a sufficient quantity of phenolized saline. The hematoxylin stained antigen, thus prepared, was bottled and stored in the refrigerator ready for use.
THE TEST

Clean glass tubes, such as are used for the agglutination test, were numbered according to the numbers of the milk samples. A 1-cc. pipette was used to measure 1 cc. of the milk from each sample bottle; and this amount was poured into a tube. Before the milk was transferred, the sample was thoroughly shaken, to mix the cream and milk. For each sample, a separate pipette was used.

One drop of hematoxylin stained antigen was added to each tube. The tubes were shaken thoroughly, and incubated for one hour at 37° C. At the end of the incubation period, the tubes were removed from the incubator, and readings recorded.

In positive cases, the stained bacteria rose to the top with the cream layer, forming a blue ring, and leaving the un-coloured milk at the bottom. In suspicious cases, the ring formation was not as distinct, and the milk remained slightly coloured. In negative tests, no ring formation took place: the milk remained as it was originally, with the yellow cream layer at the top. Plate No. 1 shows the difference in the three reactions.

The period of incubation hastens the reaction or the formation of the cream layer on top of the sample. Check tests were run on duplicate samples, to ascertain whether the period of incubation was necessary. Without incubation, or at room temperature, it was necessary to leave the samples for at least two hours to accomplish agglutination, and to have an adequate cream layer.
WHEY AGGLUTINATION TEST

From each milk sample, approximately 5 cc. were transferred to clean test tubes with a 5 cc. pipette. The tubes were numbered, to prevent error. To each 5 cc. of milk, two drops of extract of rennin were added, and these were mixed. The tubes were incubated at 37°C. for about 45 minutes, and then removed from the incubator. They were centrifuged for approximately 45 minutes, at 2000 revolutions per minute, so that the clear whey separated out, leaving the curd at the bottom.

The tests were conducted to limiting titre. For each sample, nine clean tubes were arranged on a rack. One cc. of physiological saline was measured into each tube. With use of a Cornwall syringe, one cc. of serum was diluted in 5½ cc. of saline, in a separate test tube.
From this, 1 cc. was transferred to the 1 cc. of saline in the first tube. This was mixed thoroughly, and again 1 cc. from this tube was transferred to the second tube. This process was continued to the last tube, from which 1 cc. was discarded. To each of these tubes 1 cc. of standard antigen was added, thereby making dilutions of 1:25; 1:50; 1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200 and 1:6400. The syringe was thoroughly washed between each sample. Standard antigen, which was used for the blood agglutination test, was used throughout this experiment.

The tubes were then incubated for 36 to 48 hours at 37°C. They were removed from the incubator at the end of this period, and readings recorded. The agglutination results in individual serum antigen mixture were recorded as complete agglutination by a plus sign (+), incomplete agglutination by P, and no agglutination by a minus sign (-). The following characters were used throughout:

- = No agglutination in any dilution
P = Partial agglutination in 1:25 dilution
+ = Complete agglutination in 1:25 dilution
+P = Complete agglutination in 1:25 dilution and partial in 1:50 dilution
2+ = Complete agglutination in 1:50 dilution
2+P = Complete agglutination in 1:50 and partial in 1:100 dilution
3+ = Complete agglutination in 1:100 dilution
4+ = Complete agglutination in 1:200 dilution
5+ = Complete agglutination in 1:400 dilution
6+ - Complete agglutination in 1:800 dilution
7+ - Complete agglutination in 1:1600 dilution
8+ - Complete agglutination in 1:3200 dilution
9+ - Complete agglutination in 1:6400 dilution.

**BLOOD SERUM AGGLUTINATION TEST**

The blood samples were broken down, and then centrifuged for 15 minutes at 600 revolutions per minute, to separate the serum from the clot. The test was conducted in a similar manner to the Whey Agglutination Test, using limiting titre. Readings were recorded, as already mentioned in the Whey Agglutination Test.
TABLE I

COMPARATIVE STUDY OF ANTIGENS - DANISH AND EXPERIMENTAL

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RF - Right Front Quarter  RH - Right Hind Quarter
LF - Left Front Quarter   LH - Left Hind Quarter
+ - Complete Agglutination - Positive
- - No Agglutination - Negative
P - Partial Agglutination - Suspicious
NF - The quarter of the cow was non-functioning.

Table I was prepared to show the correlation between the antigen used in this experiment and the antigen obtained through the courtesy of Doctor Jepson, of Denmark, which was used as a control. The antigen used by the Danish workers was a glycerine suspended antigen, whereas the one used in the studies reported here was a carbolized saline suspension of the hematoxylin stained Brucella.
The glycerine suspended antigen was difficult to mix with the sample of milk; unless extreme care was exercised in mixing the antigen with the milk, there was danger of some of the stained bacteria settling at the bottom of the tube. The saline suspended antigen was easily incorporated with the milk samples, and no settling was noted. Readings were more distinct when the latter antigen was used. Suspicious samples also were more distinct with the saline antigen. The results of testing 140 samples of milk from 18 cows, with two antigens, agreed in all cases. After both antigens had been thoroughly checked, it was decided to use the carbolized saline suspension of hematoxylin stained Brucella.

In order to determine the reaction of the Ring test in various dilutions, serial dilutions were made. A series of twelve tubes were arranged on a rack. Into each of these tubes one cc. of tested, non-infected milk was placed. From some of the positive Ring test samples, picked at random, one cc. of milk was transferred to the first tube with a Cornwall syringe. This was mixed with the one cc. of non-infected milk; and one cc. of the mixture was drawn into the syringe and transferred to the second tube.

The process was repeated in each case and continued to the last tube, from which 1 cc. was discarded. This gave a dilution of 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256; 1:512; 1:1024; 1:2048 and 1:4096.

To each of these tubes one drop of hematoxylin stained antigen was added; and the tubes were shaken thoroughly. The samples
were incubated for one hour at 37°C. In all, 102 samples were tested. In certain cases, where the dilution was 1 to 1024, the antigen was agglutinated and rose with the cream to the top. It could be seen that the dilution factor depended on the degree of agglutinins in the milk. Plate No. 2 shows such a serial dilution. In this plate, the dilution began at the 2nd tube and ended at the 12th. Rings were distinct up to the 10th tube, where the dilution was 1:512. In the 11th tube there was only partial ring formation; and no ring formation had taken place in the last tube. Tube 1 contained the tested sample of infected milk, undiluted.

Plate No. 2 - Serial dilutions showing the reaction of the Ring Test in the higher dilutions.

The Ring test is used extensively in the bovine brucellosis control programme in Denmark, at the present time (6). Danish officials reply, to a considerable degree, on the sensitivity of the test; their initial programme is based on the detection of brucellosis by testing composite samples.
In the author's study, determination of the reaction of the Ring Test was made, using dilutions which varied from 1:2 to 1:4096. The milk samples used contained Brucella agglutinins. It was observed that the antigen was agglutinated in different dilutions in different samples, depending on the degree of agglutinins in the milk. In certain samples, the antigen was agglutinated and formed a ring when the dilution was 1:1024; and, in other cases, ring formation had taken place only up to 1:4 dilution. This indicated that, the higher the degree of agglutinins in the milk, the greater the dilution reaction.

It would seem, from these results, that one gallon of milk containing Brucella agglutinins might be capable of contaminating 1024 gallons of normal milk. Hence, milk containing a high degree of agglutinins - from a cow which had only one quarter infected with Brucella abortus - would contaminate the milk obtained from an entire herd. This contamination could be detected by utilizing the Ring Test.

Along with this study, the percentage of positive reactions in the serial dilutions was also ascertained. The percentage of positive reactions decreased as the dilution increased. It was observed that, in a dilution of 1:128, the Ring test gave 50 percent positive reaction; beyond this dilution, the percentage suddenly dropped. Since the percentage of positive reactions decreased with the dilution, it may not be feasible to use a dilution greater than 1:128, in examining composite herd samples for the detection of
Brucella infection.

In this respect, the Ring test was found to be very useful in detecting even one shedder cow in a large herd. The test would thus prove valuable as a means of quick detection of brucellosis in a herd. A positive result from a composite herd sample signifies a focus of infection in this herd. A further test with individual composite samples discovers the shedder cow.

As the data collected in the experiment for the above determination was not supported by adequate controls, a table showing the various reactions is not included here.
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### Table IX - Continued

**Comparative Results of the Ring Test and Whey Agglutination Test on Milk Samples from Positive and Negative Animals**

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RF = Right Front Quarter, RH = Right Hind Quarter, LF = Left Front Quarter, LH = Right Hind Quarter
+
Positive, Complete Agglutination
-
Negative, No Agglutination
P Partial agglutination (Suspicious)
NF = The quarter was Non-functioning
Unfit = The sample was unfit for testing.
Table II covers 446 quarter samples of milk, from 58 cows. These samples were subjected to both the Ring test and the Whey agglutination test, to find the correlation between these two tests, and to ascertain the reliability of the Ring test as a standard milk agglutination test. Of 58 cows, only 19 were positive; milk from the remaining 39 cows was negative when tested for Brucella agglutinins.

Although the number of animals used was small, it would appear that definite conclusions may be drawn. There would seem to be a definite correlation between the results obtained from the two tests. Each animal showed a uniform reaction to both tests. Samples of milk obtained from the left front and left hind quarters of cow No. 24 remained negative in both the Ring test and the Whey agglutination test. Milk from the right front and right hind quarters of this cow was positive in the Ring test; and milk from the right hind also showed a positive reaction in the Whey agglutination test. The reaction of the sample of milk from the right front to the Whey agglutination test could not be obtained, as the sample was unfit for the test.
### Table III

Comparison of Ring Agglutination Test and the Whey Agglutination Test in Conjunction with the Blood Serum Agglutination Titre

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### TABLE III - CONTINUED

**COMPARISON OF RING AGGLUTINATION TEST AND THE WHEY AGGLUTINATION TEST IN CONJUNCTION WITH THE BLOOD SERUM AGGLUTINATION TITRE**

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### TABLE III - CONTINUED

**COMPARISON OF RING AGGLUTINATION TEST AND THE WHEY AGGLUTINATION TEST IN CONJUNCTION WITH THE BLOOD SERUM AGGLUTINATION TEST**

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<th>BLOOD AGGLUTINATION</th>
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- = No agglutination in any dilution
P = Partial agglutination in 1:25 dilution
+ = Complete agglutination in 1:25 dilution
+P = Complete agglutination in 1:25 dilution and partial in 1:50 dilution
2+ = Complete agglutination in 1:50 dilution
2P = Complete agglutination in 1:50 and partial in 1:100 dilution
3+ = Complete agglutination in 1:100 dilution
4+ = Complete agglutination in 1:200 dilution
5+ = Complete agglutination in 1:400 dilution
6+ = Complete agglutination in 1:800 dilution
7+ = Complete agglutination in 1:1600 dilution
8+ = Complete agglutination in 1:3200 dilution
9+ = Complete agglutination in 1:6400 dilution
NF = The udder was non-functioning
Unfit = The sample of milk was not fit for the test
B.I.T. = The container was broken in transit
Table III has been formulated to evaluate the Ring and the Whey agglutination tests on milk samples, in relation to the blood reaction. The table includes data on 469 quarter samples of milk from 120 cows, and on blood samples collected from the same cows. The samples of milk were tested by the Ring and the Whey agglutination tests, and the blood by the blood serum agglutination test.

From Table III, it can be seen that there is complete correlation between the results of the Ring test and of the Whey agglutination test. In all cases, cows positive to the Ring and Whey agglutination tests also proved positive to the blood agglutination test; as, for example, cows No. 1, 2, 3, 7, 24, 111 and 112. Thirty-five cows proved to be negative in all three tests; as, for example, cows No. 8, 11, 15 and 20.

The blood titres, of animals found to be positive in all the three tests, varied from 1:100 to 1:6400. Cow No. 24 had a blood titre of 1:100, and Cow No. 3 had a titre of 1:6400. Fourteen cows showed a blood titre of 1:50, as in the case of Cow No. 9; and seven cows showed a titre of 1:25, as in the case of Cow No. 36. A titre of 1:25 or 1:50 is considered suspicious, and animals with such blood titres cannot be included among positive reacting cases. These animals remained negative to the Ring and Whey agglutination tests.

It would seem from this study that animals, which might
be classified as suspicious by reason of their reaction to the blood agglutination test, nevertheless showed no agglutinins in their milk, as demonstrated by the Ring test and Whey agglutination test. Whether this would indicate that the blood agglutination test is more reliable than the Ring test, or that such animals are not really infected with brucellosis (their blood reaction being non-specific), is open to discussion. The question may also be raised as to whether such animals have recently become infected, and the blood titre is on the increase before the mammary gland becomes infected.

In 1916, Cooledge (3) stated that active infection of the udder with Brucella abortus produced antibodies in milk, and that these antibodies were not due to diffusion of antibodies from the blood. Smith, Orcutt, and Little (39) also pointed out that milk agglutinins come largely from the udder. Tweed (44) observed that milk agglutinins originated in the udder, and are not transferred from the blood stream.

In Table III, it can be seen that Cows No. 16, 115, 116, 117, 118, 119, and 120 proved to be positive to the blood agglutination test, and remained negative to the Ring and the Whey agglutination tests. This finding is consistent with the findings of the above quoted investigators. Therefore, the disagreement between the results of blood and milk tests may occur because the Brucella agglutinins of the blood are not transferred to the milk.
### TABLE IV

COMPARATIVE STUDY OF THE RESULTS OF THE RING TEST.

**WHEY AGGLUTINATION TEST AND BLOOD AGGLUTINATION TEST**

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Table IV shows some factors affecting the accuracy of the Ring and Whey agglutination tests. It was noticed, in Tables No. II and III, that there was complete correlation between the results of the Ring test and the milk serum agglutination test. In Table IV, it will be seen that, in many cases, whole milk showed a lower reaction to the Ring test than to the milk serum agglutination test. For example, samples from Cows No. 17, 18, 19, 20, and 21 failed to show a clear reading in the Ring test; whereas the corresponding readings were clear in the milk serum agglutination test.

Throughout the experiment, it was noticed that samples of abnormal milk, from mastitis-infected cows, in many cases failed to give a reliable, consistent reading in the Ring test, owing to the failure of the cream to rise to the surface. Those cases have been included in this table.

As far as the results of the milk serum agglutination test and the blood agglutination test are concerned, there is a complete lack of correlation. Both tests give varying titres, without any apparent relationship. Cows No. 22, 29, 13, and 33 show a high blood titre, with a corresponding low milk titre. On the other hand, Cows No. 7, 9, 11, and 18 show a high milk titre, with a corresponding low blood titre. Cow No. 3 was negative to the blood agglutination test, and positive in three quarters to the Ring test and the milk serum agglutination test. There is also variation in the titres of individual quarters of the same udder, as seen in cases No. 1, 14, 17, 21, and 36.
The results of these experiments would indicate that there is no apparent relation between the Brucella antibody content of the milk and that of the blood, and that the degree of agglutination depends upon the extent of infection in the particular quarter from which the sample was taken.

The efficiency of the test depends largely upon the quality of the milk samples. It is important, therefore, to collect normal milk samples, to obtain a reliable reading.
EXPERIMENTAL INOCULATIONS OF GUINEA PIGS WITH MILK FROM ANIMALS
POSITIVE OR NEGATIVE TO THE RING TEST

METHODS

The isolation of Brucella abortus from the milk of cows, by guinea pig inoculation, was first reported by Schroeder and Cotton (33). Since then the method of guinea pig inoculation has been used extensively, for the detection of shedder cows. Henry, Traum, and Haring (21), in 1932, used cream and milk sediment for guinea pig inoculation, after observing that in centrifuged milk the cream and sediment contained the larger percentage of Brucella abortus organisms. Hofstad (23), 1940-41, used cream of infected milk for inoculating guinea pigs. Milk sediment was used by Huddleson (25), in 1920, for isolating Brucella abortus.

In the writer's experiment, samples of milk were incubated for one hour, so that the cream rose to the surface. Other samples were centrifuged at high speed for 20 minutes. The cream and sediment were thoroughly mixed, and 4 cc. were inoculated into guinea pigs intraperitoneally. Male guinea pigs were used for the experiment, because Henry, Traum, and Haring (21) have stated that male guinea pigs showed characteristic lesions in the testis and seemed somewhat more susceptible than females to infection with small numbers of the organism.

After five weeks, the guinea pigs were posted. The spleen was removed aseptically, into a sterilized petri dish; and a sample of blood from the heart was removed into a sterilized test tube for the agglutination test. During autopsy, an examination
was also made for gross lesions and enlarged lymph glands. In some cases, the spleen was enlarged two to three times its normal size. Plate No. III shows the comparison between normal and abnormal spleen.

![Plate No. 3 - Abnormal and Normal Spleen.](image)

The rapid agglutination test has been used to detect Brucella agglutinins in the blood serum of guinea pigs. Plate No. 4 shows the three stages of agglutination.

![Plate No. 4 - 1. Complete Agglutination, 2. Partial Agglutination, and 3. No Agglutination.](image)
The spleens were cultured on tryptose agar slants, both aerobically and under 15 per cent carbon-dioxide tension. They were incubated at 37° C.; and growth was noted. Brucella abortus grows best under reduced oxygen tension, on initial cultures. No tubes were discarded before at least 10 days' incubation. Those showing growth were sub-cultured, to obtain a pure culture. Smears were made, to determine whether the typical colonies were made up of small gram negative rods. These were further checked with serum known to contain Brucella abortus agglutinins.
### TABLE V

**RESULT OF GUINEA PIG INOCULATIONS OF COMPOSITE SAMPLES OF MILK FROM COWS POSITIVE TO THE RING TEST**

<table>
<thead>
<tr>
<th>SAMPLE NO.</th>
<th>COMPOSITE SAMPLES</th>
<th>RING TEST</th>
<th>BLOOD TITRE</th>
<th>GUINEA PIGS INOCULATION</th>
<th>REMARKS</th>
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A review of literature showed that more than 50 per cent of infected animals were capable of shedding Brucella abortus in their milk.

Sadden (36), reported that 71 per cent of cows reacting to the agglutination test eliminated Bang's bacillus through the udder. Schroeder, and Cotton (34), showed that 83 per cent of infected cows, having a titre of 1:200 or, higher, produced the abortion.

<table>
<thead>
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B.I.T. = Container broken in transit.
bacillus in their milk. Gilman (14) stated that Brucella abortus was found in the milk from 63 per cent of cows having a titre of 1:80, or higher. Fitch and Bishop (11) inoculated guinea pigs with samples of milk which had agglutinins only in a 1:5 dilution; and they demonstrated the presence of Bang's bacillus even in milk with such a low titre.

Table VI has been formulated from the results of Table V, to show the number and percentage of positive and negative animals. Percentages were calculated on the number of cows, excluding those cows whose milk was fatal to the inoculated guinea pigs. Cows No. 49 and 78 were not included in Table VI, as their blood titres were not known. The guinea pig inoculated with a sample of milk from Cow No. 49 died; and the result of guinea pig inoculation with a milk sample from Cow No. 78 was negative.
Table VI reveals that a blood titre of 1:6400 has produced 87.5 per cent of such animals positive to guinea pig inoculation of milk, and a titre of 1:3200, 66.6 per cent. Between a titre of 1:100 to 1:1600 the percentages of positive reactors varied from 20 to 56.25. Some animals with a blood titre of 1:50, a titre which is not considered positive in the regular blood agglutination test, have shown agglutinins in their milk and have been known to eliminate Brucella abortus. In this study, out of 6 such animals, one, Cow No. 25, was found to eliminate the organism. Such an animal would be considered no more than suspicious on the routine blood test. However, the probability exists that this animal was recently infected and was developing agglutinins when tested.
It also indicates that cows with low blood titre might eliminate the organism in the milk at some period or other during the course of the disease.

The organisms were chiefly isolated from animals which were highly positive to the blood agglutination test, and also highly positive to the Ring test. Although a total of 86 cows was used in the experiment, only the data on 74 cows were available: 11 guinea pigs inoculated with milk died, and one culture was contaminated. When the milk from these 74 cows, which were positive to the Ring test, was inoculated into guinea pigs, Brucella abortus organisms were isolated from 36 animals (or a percentage of 48.6).

The percentages of animals, shown by guinea pig inoculation to be positive and negative, can be seen in Table VI. Animals showing high and low degrees of agglutinins have been found to discharge Brucella abortus in their milk. This does not preclude the chance that a percentage of the animals, which were found negative by the method of culture used in this study, may be eliminating the organism. The possibility exists that some of these animals may be shedders of the Brucella abortus bacillus, and that repeated culture might show a higher percentage of positive reactors.
Nine cows which were negative to the ring test are included in Table VII. In all cases, except one cow No. 6, guinea pigs inoculations with milk also proved negative. It is of interest to note that Cow No. 7 was negative to the Ring and Blood Agglutination Test and was found to be shedding Brucella abortus in her milk. The history showed that this animal was recently infected and perhaps had not developed agglutinins in her milk or blood at the time the samples were taken. A later test showed a positive reaction. Cows No. 4, 7 and 9 showed blood titres of 1:100, 1:200 and 1:400 respectively.

The milk did not contain Brucella agglutinins and Brucella abortus could not be isolated on Guinea pig inoculation. This may be
due to the fact that the organism does not necessarily stimulate the production of agglutinins in the milk unless the udder is infected.

The results of this table would indicate, however, that animals with a negative milk titre, as shown by the Ring test, are relatively free from actual udder infection.
SUMMARY AND CONCLUSIONS

In the writer's experiments, 1171 samples of whole milk were subjected to the Ring test, and 996 samples to the Whey agglutination test. The results agreed, in all cases, where the milk was normal. In all cases of high milk agglutination titre, the corresponding Ring test was strongly positive.

The determination of the reactions of the Ring test in varying dilutions was carried out on 102 samples of milk containing Brucella agglutinins. The reaction of milk in different dilutions depended upon the degree of agglutinins in the sample.

The Ring test can replace the Whey agglutination test in the routine testing of milk samples for Brucella abortus agglutinins. Its usefulness as an initial diagnostic test for the detection of herd infection has been definitely proven.

The efficiency of the Ring test depends on the quality of the milk sample. In cows which show abnormal milk, the Ring test may give false positive or false negative readings.

A comparative study was made on the results of the Whey agglutination test and blood agglutination test. It was observed that the antibody content of milk differed from quarter to quarter of the same udder. Furthermore, there were cases where one or two quarters remained negative, and the others were positive. Cases have also come to notice, in which, the animal was negative to the blood agglutination test and positive to the Whey agglutination and Ring tests. It was therefore concluded that there was no apparent
connection between the Brucella antibody content of the blood and that of the milk.

In all, 124 guinea pigs were inoculated with positive or negative composite samples of milk, for the isolation of Brucella abortus. Of 74 composite samples of milk, which contained Brucella agglutinins as shown by the Ring test, 36 samples (or a percentage of 48.6) yielded Brucella abortus through guinea pig inoculations.

In most instances, the larger percentage of positive milk was found in cases where the blood agglutination and Ring test results were both high.
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C 361
1948

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