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The Brucella Ring Test for Milk of Individual Cows and its Value for Determining their Status of Infection.

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EXAMINATION of milk for brucellosis by antigen-antibody reactions has recently been brought into the scope of the practising veterinarian by the application of Fleischhauer and Hermann's "Abortus Bang Ringprobe." This is a test for bulk milk samples and coloured antigen is used (Hermann 1937, 1939, Fleischhauer 1937, 1939). This test is used in Sweden and Denmark for the control of *Brucella abortus* infection (Norell and Olson, 1943; Christiansen, 1948; Bruhn, 1948). Although the rapid blood agglutination tests for brucellosis in cattle and salmonellosis in chicks are not very accurate, they still have considerable value in field work. The Brucella Ring Test is a practical field test and in simplicity it even surpasses the agglutination tests mentioned.

In dairy farming areas in South Africa the benefits obtained from the use of this test have not yet been exploited. The purpose of this report is to record some experiences in the use of the test on individual animals. It is desired to draw particular attention to a hitherto unrecorded difference in the Ring Test reaction of milk from infected cows and milk from vaccinated cows. Both types of cows react to the blood serum agglutination test without readily demonstrable differences in results.

Norell and Olsen (1943), Bruhn (1948), and others have reported work on the value of the Ring Test in Sweden and Denmark. The orginal results of Hermann (1937, 1939) and those of Fleischhauer (1937, 1939) have been substantiated. The test has even been acclaimed as a new method of controlling Contagious Abortion [The North Am. Vet. (1948), 29: 488; Hoard's Dairyman (1949) 94 (3): 130]. Modifications in technique of antigen production and standardisation to improve the specificity and reliability of the results have been recommended by Bruhn (1948). Wood (1948) has found the test reliable, with fewer sources of error than the whey agglutination test and gives details of a rapid method for the production of antigen.

A preliminary survey on a small scale in South Africa supplied very interesting data on the Brucella antibody content of the local milk supplies. Knowing the small amount of vaccine applied in the districts concerned, it was possible to conclude that well over 50 per cent. of herds were infected with brucellosis (v. Drimmelen (1948, 1949). A contemporary investigation on the whey agglutination titres of a similar group of milk supplies [Lewin, Bersohn and Richardson, (1948)] shewed comparable results. These authors expressed doubts about the extent of Brucella infection in milk giving positive whey agglutination titres. The relatively

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small number of milk samples shewing infectivity for guinea-pigs appeared to them to suggest that most reacting milks might not actually be infective. In a previous report (v. Drimmelen, 1948) the rapid loss of infectivity of grossly infected milk from individual cows was indicated. It is believed that bulk milk samples containing one part of infected milk to several parts of Brucella-free milk, would require very large doses of properly prepared milk or cream to demonstrate its infectivity for guineapigs. If the milk is not quite fresh the biological test is probably not very reliable. In this respect the biological test of milk is less useful for brucellosis than for tuberculosis. Pullinger (1934) found the whey agglutination test more useful than the biological test for the detection of brucellosis in contaminated milk samples. The same author showed, however, in a later report (Pullinger, 1936) that 18 hours storage of milk contaminated with saprophytes did not destroy Brucella organisms in spite of a drop in pH to 4·8. He also found the organisms in cream and cheese after long periods (Pullinger, 1935).

Distinguishing infected cows which are potential "spreaders" of virulent Brucella organisms, from reacting cows which are not "spreaders" but which usually have a high degree of resistance to infection, has been attempted by means of the whey agglutination test (Scrivner, 1937; Huddleson, 1942; Traum and Maderious, 1947). Different titres for whey have been associated with local udder infection [e.g. 1:25 (Traum and Maderious, 1947) or 1:80 (Wilson and Miles, 1946)]. Variable results obtained from a single sample with varying rates of clot retraction have, however, demonstrated the unreliability of this test (Wood, 1948). A herd may show negative whey reactions in 50 per cent. of cows with infected udders (Huddleson, 1943). According to Davies (1947) Brucella organisms may be excreted in milk with a negative whey agglutination titre, (Wilson and Miles, 1947; Davies, 1947) or a negative blood serum agglutination titre (Karsten, 1932).

The effect of vaccination on the agglutination titre of infected cows has been found to differ from the effect of vaccination on the reaction of cows which have lost their infection. The infected cows show no change in the titre, whereas in cows which have lost the infection, a previously stabilised or receding titre would be raised by the 17th day following vaccination. Promising results with this method have been reported by Dick, Venzke and York (1948). Our own limited experience has not given reliable differential blood serum titres with this test.

American workers attach the greatest importance to the positve serum agglutination reaction in vaccinated adults because it is a factor which interferes with the control programme adopted in the United States. If the Brucella Ring Test on the milk should give a fairly reliable result at a known time interval after vaccination it would be an invaluable aid in dealing with both persistent reactors free from udder brucellosis and such potential spreaders as the "ceased" reactors to the blood serum agglutination test.

Several Brucella Ring Tests were carried out on the milk of individual cows because the results on bulk samples were complicated by the unknown factor of dilution. A striking difference between the reactions of infected and vaccinated animals was noticed immediately. By slightly modifying the methods of performing the test, this difference could be clearly demonstrated.

MATERIALS.

The antigen used for the test has been described in detail in previous reports (v. Drimmelen, 1948, 1949). Though Hermann (1937) found it only suitable for use within a few weeks our antigen which was prepared on the lines of Brucella vaccine Strain 19 (Buck) grown on potato agar and harvested in an almost closed

system to exclude contamination, showed constant results for several months. The antigen was stored at 2-3°C. Since the publication of Bruhn's modifications to the test these were introduced here but the glycerinated antigen had to be discarded again because the results were not as distinct.

The milk used for testing was collected from experimental and control animals kept in isolation boxes and camps and milked only once per day. They were predominantly beef type, (e.g. Sussex × Africander). Milk samples were collected in one or more 10 c.c. bottles filled from the last strippings and containing milk from all four quarters in approximately equal quantities.

Methods.

The technique of the test as originally described was altered to include dilutions of the reacting milk with milk of the negative control animal. In this way it was possible to describe the strength of the reaction in a numerical figure depending on a constant mechanical factor only. At first the dilutions 1:2·5, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 were used but the more satisfactory 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512 were later adopted. This test eventually consisted of ten tubes.

One ml. negative and creamy milk was filled into the nine last tubes. One ml. of the milk to be tested was placed into first (empty) tube and a further one ml. added to the second (containing a ml. of negative milk). The contents of this second tube were mixed and the dilution was continued serially by transferring one ml. diluted milk to every one of eight successive other tubes. One drop coloured antigen suspension was added to each tube and they were incubated at 37°C. for 40-50 minutes and read immediately.

Final Technique.

The technique was finally used in fieldwork and for practical purposes developed into a simple, clear and rapid field test. This test only requires the coloured antigen and three clean test tubes per animal. Sterilization of the tubes is *not* necessary. One ml. of fresh creamy milk from a known negative cow is placed in each of two tubes. One ml. of the milk from the animal to be tested is placed in the remaining tube and another one ml. of this milk is added to negative milk in one of the tubes containing negative milk. This is mixed (1:2) and one ml. of the mixture is transferred to the remaining tube containing fresh negative milk. Here it is again mixed (1:4) and one ml. of this mixture is discarded. The whole operation can be done with one syringe or pipette. The amount of one ml. is arbitrary and approximate but it must be the same throughout the one test. The syringe or pipette may be cleaned by rinsing once in cold water and once in hot water between tests. A drop of coloured antigen is added to each tube which is then vigorously shaken and incubated in a waterbath for 40 minutes or in a still air incubator for 50 minutes at 37°C. or 98·4°F. (If necessary a bucket can be made to serve as a waterbath, and a vest pocket may serve as incubator for a small number of samples).

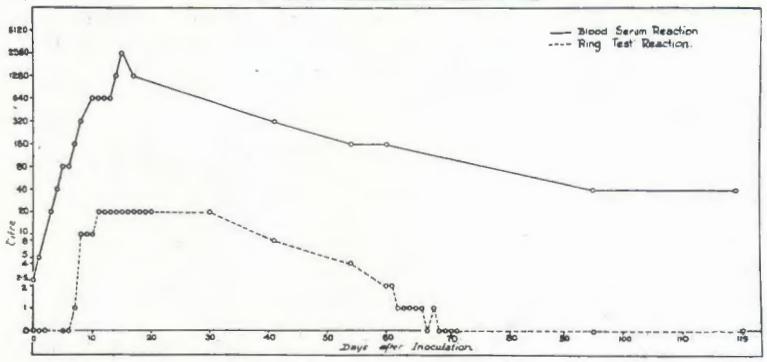
Results.

1. *A vaccinated cow in milk.*

A cow (No. 7913) was vaccinated about one month after calving. The vaccine used was strain 19 (Buck) live avirulent *Brucella abortus*. A duplicate count was performed to show that 58,000,000,000 viable organisms were inoculated subcutaneously on the left side of the neck. Biological tests were carried out daily at first and weekly later on, to prove that this cow did not secrete Brucella organisms in the milk.

Graph (*a*) shows the results of vaccination of cow 7913. A typical blood serum agglutination response and the corresponding Ring Test results are shown. *The Ring Test reaction was only noticed during the height of the serum agglutination reaction.*

Graph (a) : Showing the Comparison of the Ring Test Citre and the Blood Serum Citre in a vaccinated Cow.



2. *A dry cow vaccinated with S.* 19.

By daily udder massage a sterile dry cow (No. 7912) was made to supply a small quantity of serous fluid. After this cow was vaccinated no Ring Test could be carried out on the fluid, but the coloured antigen was observed to agglutinate and sediment to the bottom. This suggested the addition of cream from a negative cow to the fluid. When this was done, a distinct and positive Ring Test reaction was produced. By adapting the serial *dilution technique* it was shown that the development and recession of the reaction proceeded similarly to the one in graph (*a*).

3. *A non-vaccinated cow drying off.*

A cow (No. 6933) had been used as a negative control. The animal had to be dried off when she neared full term. Her udder secretion was, however, tested daily by the Ring Test to see the effect of drying-off secretion on the result of the test. An indistinct Ring Test reaction was obtained though she never gave a distinct positive in the pure drying-off milk. A 1:1 dilution with milk of a negative cow also gave suspicious coloured bands near the top on some days. The higher dilutions tested by the serial dilution technique were, however, uniformly negative. The milk was of course thick, yellowish and of high cellular content, yet the observation definitely showed a deviation from the negative in an animal free from all antigenic stimulation with Brucella organisms. Only by *the dilution technique* was it possible to get a clearly negative result.

4. *An infected cow in milk.*

An animal used as positive control in the earlier Ring Tests (cow No. 9096) was about two years in milk without having been served. Her blood serum agglutination titre was gradually receding although biological tests on the milk proved her to be a regular " spreader " of Brucella organisms. The application of the serial *dilution technique* revealed a very high Ring Test titre in contrast to the

relatively low blood titre in this animal. The importance of this was immediately realized. As a result all available relevant data were collected and analysed with reference to the differences between vaccinated and infected reactors.

The attempts made to compare the results of the Ring Test on a number of cattle at this laboratory with other tests for Brucellosis are based on the data condensed in table 1 and listed fairly fully in tables 1 (*a*), 1 (*b*), 1 (*c*), and 1 (*d*) which are appended.

TABLE 1.

Comparison of the Ring Test reaction, the blood serum agglutination test, the biological test and the cultural tests in vaccinated and infected cows.

Class of Cows.	Time Interval after Vaccination or Infection.	Ring Test Reactions.	Agglutination Tests.	Biological Tests.	Cultural Tests.
Vaccinated.....	0– 2 months	+++	+++	+	+
	3– 4 months	++	++++	+	–
	5– 6 months	No Reaction.	+++	+	Nil
	7– 8 months	No Reaction	+++	Nil	Nil
	9–10 months	No Reaction	++	Nil	Nil
	11–12 months	No Reaction	+	Nil	Nil
	1 year or more	No Reaction	+	Nil	Nil
Infected.....	0– 2 months	+++	+++	++	++
	3– 4 months	++++	++++	+++	++
	5– 6 months	+++	+++	++	++
	7– 8 months	+++	+++	++	Nil
	9–10 months	+++	+++	Nil	–
	11–12 months	+++	+++	+	Nil
	1 year or more	+++	+++	+	Nil

++++=All positive. +=Some positive.
+++=Most highly positive. –=None positive.
++=Many positive. Nil=No tests carried out.

These data suggest that a very marked advantage is to be obtained from the use of the Ring Test in individual cows.

The test quickly indicates all highly positive reactors but low residual titre, such as may be found in the blood serum six months or even years after vaccination, does not show up by the Ring Test in dilutions.

Carriers of Brucella infection which usually spread the organisms through the milk, show a markedly positive Ring Test reaction even if the titre of the blood serum has dropped to a fairly low level.

The Ring Test is reliable in that it never failed to give a reaction in infected animals a week or more after inoculation. Unlike the cultural and biological tests, contaminants of the milk are unable to interfere with the results in the time required for the Ring Test. There is no delay in obtaining the results of the Ring Test.

Where only 60 per cent. or less of infected animals can be spotted by the cultural and biological test the Ring Test shows up almost 100 per cent.

The differences found are clearly shown in graph *b*, which is based on the data given in the appended tables.

Discussion.

The first question arising in connection with any proposed method of distinguishing vaccinated and Brucella infected cows is whether it is a method that can be applied economically. The Ring Test appears to have a promising future because it is easily performed and practical.

Briefly the difficulties associated with the earlier tests, serological, cultural and biological, are the following:—

(*a*) *Cultural* examination of blood, tissues, secretions and excretions requires the highest technical skill and expensive equipment, media and much time (Huddleson, Hasley and Tarrey, 1927). It can only be carried out on an experimental scale and at proper laboratories.

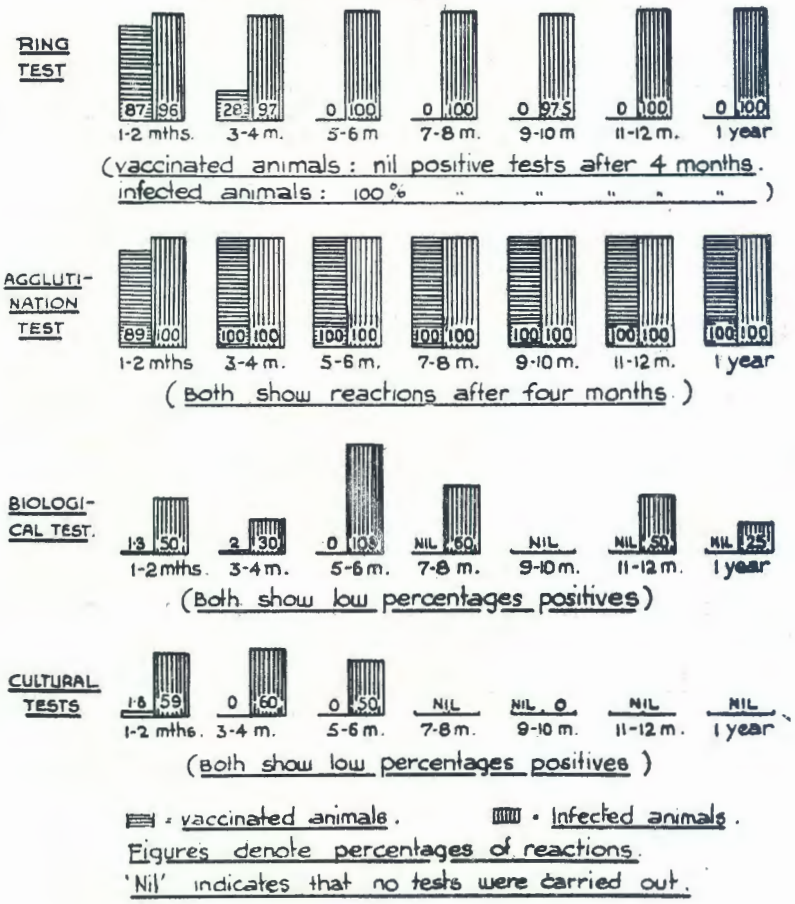
(*b*) *Biological* tests of blood and secretions by guinea-pig inoculation are very informative and constitute the surest way of arriving at a definite opinion about the presence of infection. The tests are, however, very expensive and the results can only be collected after about two months. Intermittently infected animals give negative results, (Huddleson, 1934, 1943; Pullinger, 1934, 1936, 1948). This makes these tests of little practical value in dealing with a commercial herd in the field.

(*c*) *The blood serum agglutination* test is the most common diagnostic test for brucellosis. It is of course an indirect test and to distinguish vaccinates from infected animals it is necessary to determine the titre at a number of successive tests. Even when this information is available there is no way of being absolutely certain of the presence of infection. The blood serum titre of infected cattle often tends to recede very markedly without reduction in infectivity of the milk (Huddleson, 1943). Negative reactors may eliminate infection (Karsten 1932). The rapid plate test is not very suitable for titration and in the laboratory tube agglutination tests some delay in reporting results is an unavoidable drawback. Much recording and clerical work is required.

(*d*) *The Whey Agglutination test* was recommended by Traum and Maderious (1947) for the purpose of differentiating between infected and non-infected reactors. It will no doubt play a rôle in handling of suspected cows. The test requires much glassware and space and is definitely a laboratory procedure. The comments of the previous paragraph on the tube agglutination test thus apply. Wood (1948) has shown, however, that the whey agglutination titre depends upon the speed at which the clot contracts when the whey is prepared.

(*e*) *The Blood Serum Titre differential test* after vaccination is a method recommended by Dick, Venzke and York (1947) and Venzke (1948). It requires two titre determinations which may, however, be done by the rapid plate agglutination method. Then the animals with a stabilised or receding titre may be vaccinated and the reactors which show a significant rise in titre on the 17th day after vaccination can be considered free from infection. This test has the advantage, that it can be applied to animals not in milk but it is admittedly a lengthy procedure.

GRAPH (b) : The difference between diagnostic tests and the difference between the results of the tests in vaccinated and Brucella infected cattle.



The limited observations reported here suggest that the simple Ring Test on milk when slightly modified or elaborated to include the dilutions of 1:2 and 1:4 with fresh creamy milk, will provide a very practical way of dealing with the cows on the spot in an hour or two. If it is desired to eliminate the " spreaders " from a vaccinated herd, all animals in milk vaccinated more than four months previously may be tested and the reactors isolated immediately.

The simple technique described as the final technique under " methods " (*vide supra*) can be used under field conditions.

Positive reactors are probably all potential " spreaders " or may become " spreaders " after parturition. If doubt exists or the evidence is considered insufficient, the older methods only need be applied to them. This will save the trouble of examining all the animals reacting negatively to the Ring Test.

Some interest in the question of local production of anti-bodies in the udder is aroused by the results obtained with the Ring Test on milk. Traum and Maderious (1947) have demonstrated the marked concentration of agglutinin in the udders of infected cows. The typical Brucella infection in cattle is probably an intermittent bacteraemia with few organisms in the circulating blood stream. Small foci of chronic and subacute mastitis only visible microscopically (Runnels and Huddleson, 1925) maintain the infection. The empty uterus does not. In the pregnant uterus infection of the placenta occurs. How this takes place is not known but it is believed that the organisms are commonly found in clusters inside the polymorphonuclear cells. It appears possible that they may actually be transported inside these cells.

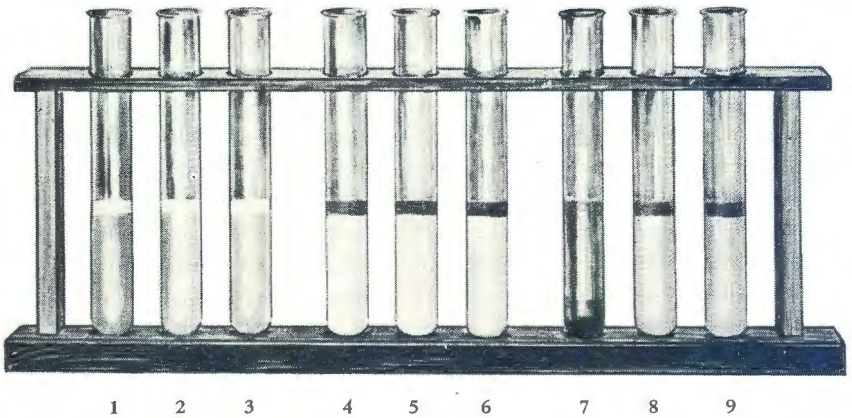


FIGURE 1.—The appearance of Ring Test results: tubes Nos. 1-3, negative milk (white cream and purplish milk); tubes Nos. 4-6, positive milk (purple cream, white milk); No. 4, pure; No. 5, 1 in 2, No. 6, 1 in 4; tubes Nos. 7-9, positive udder secretion; No. 7, no cream, purple sediment, No. 8, 1 in 2, No. 9, 1 in 4.

If cells of the lymphocytic series can elaborate the agglutinating antibodies (Harris, Grimm, Mertens, and Ehrich, 1945) the formation of large amounts of antibody in the udder and its secretion in milk may be much enhanced during a subacute inflammatory state in the alveoli. This suggests a further source of agglutinating antibody in the milk. At present the nature of the Ring Test reaction is not fully understood (Fleischhauer 1937, 1939; Hermann 1937, 1939; Bruhn, 1948). The Schern-Gorli reaction which produces a ring coloured by animal charcoal or guinea-pig red cells in the cream is believed to be involved. Agglutination has also been held partly responsible. The rising cream acts as a filter or sieve preventing the clumped bacilli from sinking and carrying them up in the cream layer. Remember that the Ring Test is read at 40 minutes whereas the tube agglutination test (Agglutinins) is only read at 20 or 40 hours. Huddleson (1943) has shown that Brucella organisms of infected cows tend to concentrate in the cream layer of milk on standing.

The outstanding feature is the finding that Ring Test results appear to show a smaller margin of doubt than any of the others. With proper technique, almost all reactions discernible are to be considered positive. This is particularly true if dilutions of the milk to be used with fresh negative milk (1:2) and (1:4) give an equally strong reaction as the pure sample to be tested. Unless a reaction is distinct, no positive diagnosis can be given. As with other tests an animal may react partially in the pre-reactive state or in the incubative stage of infection.

TABLE 1 (*a*).

The Ring Test Reaction in Milk from Vaccinated and Infected Cows.

Months after Vaccination or Infection.	Vaccinated Cows.				Infected Cows.			
	Number of Tests.	Average Titre.	Maximum Titre.	Percentage Positive.	Number of Tests.	Average Titre.	Maximum Titre.	Percentage Positive.
1– 2	105	11·4	1:20	87	28	1:36·8	1:128	96
3– 4	25	0·26	1:2	28	63	1:54·4	1:128	97
5– 6	21	—	—	Nil	34	1:48·1	1:128	100
7– 8	11	—	—	Nil	44	1:56	1:100	100
9–10	2	—	—	Nil	42	1:41·9	1:128	97
11–12	7	—	—	Nil	5	1:32	1:32	100
12 and over	5	—	—	Nil	4	1:32	1:32	100

TABLE 1 (*b*).

The Blood Serum Agglutination Reaction in Vaccinated and Infected Cows.

Months after Vaccination or Infection.	Vaccinated Cows.				Infected Cows.			
	Number of Tests.	Average Titre.	Range of Titre.	Percentage Reacting.	Number of Tests	Average Titre.	Range of Titre.	Percentage Reacting.
1– 2	83	1:563	1:2·5 to 1:2900	89	15	1:859	1:2·5 to 1:40,000	100
3– 4	29	1:68	1:10 to 1:640	100	10	1:4,350	1:1,280 to 1:10,000	100
5– 6	12	1:44·3	1:5 to 1:80	100	2	1:1,600	1:640 to 1:2,560	100
7– 8	6	1:11	1:5 to 1:80	100	7	1:2,090	1:326 to 1:10,000	100
9–10	3	1:11·3	1:5 to 1:40	100	3	1:400	1:160 to 1:320	100
11–12	3	1:6·6	1:5 to 1:10	100	2	1:320	1:320	100
12 and over	6	1:5·8	1:5 to 1:10	100	6	1:320	1:320	100

TABLE 1 (*c*).

Biological Test on Vaccinated and Infected Cows.

Months after Vaccination or Infection.	Vaccinated Cows.		Infected Cows.	
	Number of Tests.	Number Positive.	Number of Tests.	Number Positive.
1– 2	77	1	62	32
3– 4	51	1	47	18
5– 6	19	0	1	1
7– 8	—	—	20	12
9–10	—	—	—	—
11–12	—	—	2	1
12 and over	—	—	16	4

TABLE 1 (*d*).

Cultural Tests on Vaccinated and Infected Cows.

Months after Vaccination or Infection.	Vaccinated Cows.		Infected Cows.	
	Number of Tests.	Number Positive.	Number of Tests.	Number Positive.
1– 2	64	1	22	13
3– 4	26	0	15	9
5– 6	19	0	14	7
7– 8	—	—	—	—
9–10	—	—	2	0

If negative contacts are present it may be advisable to do two or more tests at 21 day intervals. The advantage of the Ring Test method over the older methods is particularly obvious in this resepct. Whereas in the past the position with regard to infection in the herd could often vary considerably during the period of time between the taking of specimens and the receipt of the result, a Ring Test result is available about one hour after sampling.

SUMMARY.

1. A technique is described whereby the Brucella Ring Test for milk can usefully be applied to individual cattle.

2. Results are reported which suggest that the Ring Test on individual animals may give a very useful indication of the real status of infection in a cow (except when she was vaccinated within three to four months previously; or infected within a few weeks previously).

3. Advantage of a test which requires little technical skill and of which the results are available within an hour are pointed out.

4. The test previously utilised for the determination of infection in cattle herds are briefly reviewed.

5. Views on the mechanism of the Ring Test Reaction and its relation to antibody production in the udder are recorded.

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