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A whey complement fixation test. Its relation to whey agglutination and isolation of *Brucella abortus* from the milk of individual cows

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The Milk Ring Test (MRT) (Fleischhauer, 1937) has been widely used for 30 years as a screening test to detect brucella-infected herds. At the present time the identification of the infected individual animals within these herds is of considerable interest because of the eradication policy announced by the British Government (Hansard, 1966), the voluntary attempts to eradicate brucellosis from dairy herds particularly by the producer-retailers, and the Ministry of Health's indication that the existing legislation against the sale of raw untreated brucella-infected milk should be more vigorously applied (Min. of Health Circular 17/66).

In the early stages of the British eradication scheme it is proposed that a herd must show three negative milk ring tests at not less than three-monthly intervals before it may be registered as a 'Supervised Herd'. Thereafter full registration will follow blood testing of every animal in the herd.

In the meantime, to safeguard the health of the public, the local health authorities have neither the facilities nor the authority to take blood samples from cows and are limited solely to sampling milk. There is a need, therefore, for an adequate test for the routine examination of individual cow's milk, the prerequisite of any such test being that it should leave the least area of doubt whether the animal is infected or not. In our opinion published work suggests that the MRT is not an adequate test for the examination of individual milk samples. However, there are four quantitative serological techniques for the examination of milk which have proved to be of value in detecting brucella infection of the udder. These are:

(i) The whey agglutination (AG) test (Smith, Orcutt & Little, 1923; Traum & Maderious, 1947).

(ii) The milk plate agglutination test (Blake, Manthei & Goode, 1952).

(iii) The whey plate agglutination test (Cameron, Kendrick & Merriman, 1956).

(iv) The quantitative MRT (Ferguson & Robertson, 1960).

Kerr, Pearson & Rankin (1959) made a major contribution by showing that the udder is capable of producing antibody in response to the introduction of brucella antigens into one or more quarters, thus demonstrating the local production of antibodies. They showed that, at the beginning and at the end of lactation, serum globulins are present in milk. Therefore, depending on the stage of lactation, whey AG antibodies in milk could have arisen either from local production in an infected udder or they could be serum globulins from the blood.

In an unvaccinated animal or in an adult animal vaccinated in calfhood (5–8 months old) the presence of agglutinins in the whey at any stage of lactation is indicative of infection, either local or general. After vaccination of an adult cow, irrespective of the stage of lactation, agglutinating antibodies are present in whey for 3 months but persist in the blood for at least 2 years. In the animal vaccinated as an adult—and very often the vaccination history of an animal is not available—the presence of agglutinating antibodies is not necessarily indicative of infection.

The four tests listed above detect agglutinating antibodies and consequently cannot distinguish between adult vaccination and natural infection. In acute human brucella infections we have found that complement-fixing antibodies in the blood reflect the activity of the disease. We have been unable to find a published reference to a whey complement fixation (CF) test for brucella antibodies and it was decided to investigate this test with milk from individual cows, and to compare the results with those depending on whey agglutination and isolation of the organism.

METHODS AND MATERIALS

Milk ring test

All samples were screened by the MRT and the results were graded + + +, + +, + and − according to the standards set down by a Working Party of the Public Health Laboratory Service (PHLS) (Report, 1956).

Whey tests

Whey was prepared from whole milk using the method recommended by the PHLS Working Party (Report 1956).

Agglutination (AG)

The AG test was performed on whey dilutions (using 0·4% phenol saline as diluent) ranging from 1/10 to 1/640 with both *Brucella abortus* and *B. melitensis* antigens (obtained from the Standards Laboratory, PHLS). The dilution of whey showing 50% agglutination was taken as the endpoint of the titration.

Complement fixation (CF)

The CF test was performed as described by Bradstreet & Taylor (1962), using the method of 'long' (overnight) fixation. The antigens used were the Standards Laboratory agglutinable suspensions. They were washed free of phenol and resuspended in veronal buffer. The optimum antigen dilution was obtained from a chessboard titration against a whey of known antibody content. The complement is titrated in the presence of antigen and a 1/10 dilution of pooled negative whey, using 1·2 units of complement in the test. Usually this combination has an anticomplementary effect, so the unit of complement determined by this method does not have any relation to the 1 MHD (minimal haemolytic dose) determined by the traditional method of titration of complement in the presence of saline. In practice the 1·2 units are approximately equal to 2 MHD but the relationship between the two varies with the batches of reagents used (Farrell & Robertson, 1967).

Culture

Culture of MRT-positive individual cow samples of milk was made from overnight gravity cream (Huddleson, Hasley & Torrey, 1927) on two different selective agars—Mair's medium (Mair, 1955) and a modification of Morris's medium (Morris, 1956). The identification of organisms isolated was confirmed using the criteria laid down by FAO/WHO Expert Committee on Brucellosis (Report. 1964*a*).

RESULTS

Of the 293 MRT-positive individual milk samples which were examined 142 were MRT + + +, 60 were MRT + + and 91 were MRT +. Three hundred MRT-negative individual milk samples were also examined as controls by the whey AG and CF tests, and 4/300 (1 %) contained CF antibodies.

Table 1. *The relation between MRT, AG and CF antibodies in whey from* 293 *MRT-positive cows*

	MRT		
	+ + +	+ +	+
Total	142	60	91
No. both AG and CF positive	120 (84)	29 (50)	19 (20)
No. AG positive only	12 (8)	7 (11)	3 (3)
No. CF positive only	4 (3)	3 (5)	2 (2)
No. both AG and CF negative	6 (4)	21 (34)	67 (73)

Figures in parentheses are percentages.

Three hundred MRT-negative individual samples yielded 4 (1 %) positive CF tests (see text).

Table 2. *A comparison of the isolation rates for* Brucella abortus *obtained on culture from MRT-positive, whey AG-positive, and whey CF-positive milks*

	MRT					
	+ + +	No. of isolations	+ +	No. of isolations	+	No. of isolations
MRT-positive	142	95 (67)	60	20 (33)	91	9 (10)
AG-positive	132	91* (71)	35	17* (57)	22	7* (41)
CF-positive	124	95 (76)	32	20 (63)	21	9 (43)

* There were nine isolations when the whey AG was negative (Table 4). Figures in parentheses are percentage isolation rates.

In Table 1 the results of MRT, AG and CF tests on the 293 MRT-positive milk samples are shown. From these results it is evident that the number of MRT-positive milk samples in which whey AG and CF antibodies could not be demonstrated increases as the intensity of the MRT reaction decreases, i.e. MRT + + + 4 %; MRT + + 34 %; and MRT + 73 %. This would appear to suggest that many MRT + + and MRT + reactions on individual milk samples are in fact false positive results.

The isolation rates of *B. abortus* are shown in Table 2 for MRT-p ...e; MRT and whey AG-positive; MRT and whey CF-positive milk samples ...ese are subdivided according to the intensity of the MRT reaction.

Table 3. *The relationship between whey CF titre and iso ... of* Brucella abortus *in* 293 *MRT-positive individual milk ... les*

Whey CF titre	MRT +++	MRT ++	MRT +	Tot
⩾ 640	5/5	2/3	0/0	7/8 (
320	11/14	2/3	0/1	13/18
160	24/25	0/1	1/2	25/28
80	14/21	3/6	1/2	18/29
40	17/22	1/3	0/0	18/25
20	12/18	5/5	4/7	21/30
10	12/19	7/11	3/9	22/39
< 10	0/18	0/28	0/70	0/11(

All titres expressed as reciprocals of the whey dilutions.
Figures in parentheses are percentages.
The figures represent number of positive cultures/total nun ...ultured.

Table 4. *The relationship between whey AG titre and iso ... of* Brucella abortus *in* 293 *MRT-positive individual milk ... les*

Whey AG titre	MRT +++	MRT ++	MRT +	Tot
⩾ 640	29/33	2/3	0/0	31/36
320	21/26	2/3	0/1	23/30
160	24/32	3/7	3/5	30/44
80	9/17	4/8	2/6	15/31
40	8/19	2/5	1/5	11/29
20	0/5	4/9	1/5	5/19
< 20	4/10	3/25	2/69	9/10⁴

All titres expressed as reciprocals of the whey dilutions.
Figures in parentheses are percentages.
The figures represent number of positive cultures/total nun ...ultured.

It is evident that the correlation between the MRT and iso ...n rate is increased if the presence of either whey AG or CF antibodies is tal ...nto account. The correlation between the whey AG test and isolation rate fo ... three MRT categories (+++, ++ and +) was 71, 57 and 41 % respectivel ...mpared with the correlation of 76, 63 and 43 % respectively between whey ...nd isolation rate.

The serological test giving the highest correlation with is ...on rate was Complement Fixation and it is important to note that *B. abortus* ...solated only from MRT-positive milk when whey CF antibodies were detecte ... opposed to nine isolations from milk which was whey AG-negative. These n ...ilk samples were MRT and CF positive.

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The relationship between the titre of the CF antibody and the isolation rate is shown in Table 3 for the three grades of the MRT reaction.

B. abortus was not isolated from any of the 116 MRT-positive milk samples without detectable brucella complement-fixing antibody.

The relation between whey AG titre and isolation is shown in Table 4. It is evident from Tables 3 and 4 that the relation between the isolation rate and CF titre remains constant from 1/20 upwards, and even at the lowest level (1/10) at which CF antibody was detected a 54% isolation rate was obtained. This contrasts with the whey AG test where there is a progressive decrease in the isolation rate from 86% at a titre of 1/640 to 26% at a titre of 1/20.

DISCUSSION

This investigation shows that the presence of whey CF antibodies strongly suggests a brucella infection within the bovine udder, for *B. abortus* was isolated from 124 of the 293 MRT-positive milks and in each culture-positive milk whey CF antibody was present. Although the whey CF showed a slightly higher correlation with isolation as compared with the whey AG test the difference between the two is not appreciable (Table 2). But the CF test is a more specific index of infection than the AG test because *B. abortus* was not isolated from whey CF-negative samples, whereas it was isolated from nine milk samples which were whey AG-negative.

The whey AG test has been widely used by veterinary bacteriologists, who have reported isolation of *B. abortus* from whey AG-negative milk samples. The working density of antigen used by the veterinary bacteriologists (Weybridge Abortus antigen) is optically 6·75 times as dense as that used for the examination of human serum (Standards Laboratory, PHLS). Because of its greater density the Weybridge antigen is less sensitive for the detection of the brucella antibodies than the PHLS antigen (Farrell & Robertson, 1967). We have isolated *B. abortus* from some milk samples which were whey AG-negative even when the more sensitive PHLS antigen was used in the test.

The biotypes of *B. abortus* have the antigenic structure of either abortus or melitensis. The abortus antigen is predominant in biotypes 1 and 2, whereas the melitensis antigen is predominant in biotypes 5 and 9. These four are the most common biotypes in Great Britain, and biotypes 5 and 9 are present in 12% of the infected herds in the north of Lancashire (Farrell & Robertson, 1967). For this reason both abortus and melitensis antigen are used in the whey tests. This is of particular importance in animals which are at an early stage of infection when, although *B. abortus* is excreted in the milk, the level of CF antibody is often low. Moreover, in infections with biotypes 5 and 9 melitensis CF antibodies may be present before the abortus CF antibodies. The converse is true in infections with biotypes 1 and 2. Because of the low levels of CF antibody in the initial stages of infection it is necessary to make the CF test as sensitive as possible while retaining specificity.

In 94 (32%) of the 293 MRT-positive milk samples examined brucella antibodies

could not be demonstrated by either the whey AG or CF tests, but 67 of these were only MRT +, and a further 21 were MRT + +. A further 22 were whey AG-positive but whey CF-negative.

This investigation indicates that brucella organisms are not excreted in the milk of whey CF-negative animals, irrespective of the MRT or whey AG results. When the whey CF test is negative but the MRT and/or whey AG test is positive the animal may be in the early stages of an udder infection. In a further investigation (Robertson & Farrell, to be published), the lactating animals from a large calfhood-vaccinated herd were examined on seven separate occasions over a 10-month period and 43 cows were found to be excreting *B. abortus*, biotype 5 in their milk. In this study a number of cows were seen whose milk was MRT-positive and whey AG and CF negative at first, but became positive to all three tests 2 months later. In such cows the disease showed a steady progression thereafter, as indicated by an increase in whey AG and whey CF titres.

Our experience in this and in other instances has been that any animal which was in the early stages of infection has given a whey CF-positive test on a further sample taken 2 months later. If these two successive whey CF tests are negative, irrespective of the MRT or whey AG result, we suggest that the animal can be regarded as free from udder infection. But this does not exclude the possibility of another focus of infection which can only be determined by blood testing.

It was estimated that 36,500 cows had udder infections due to brucellosis in the British Isles in the year 1960/61 (Report 1964*b*). In north-west Lancashire there are approximately 900 producer-retailers, 20% of whose herds are known to be infected (Robertson, 1967). In attempts to safeguard the health of the public, milk from these producer-retailers is sampled frequently by local authorities and examined for the presence of tubercle bacilli and *B. abortus*. The isolation of *B. abortus* from a sample of herd milk is usually followed by a Pasteurization Order in accordance with the Ministry of Health Circular (17/66). The detection of the infected animals within these herds is difficult, as local authorities have neither the authority nor the facilities to take blood samples from the suspect animals. This limits testing to milk samples; the MRT followed by either direct culture or biological examination of all MRT-positive milk samples is impracticable when large numbers of individual samples have to be examined. For the success and smooth working of an eradication programme, the examination of individual samples by the MRT only is not advisable because it may be concluded from the present study that up to 32% of MRT-positive milk samples are not due to brucella infection of the udder.

The limitations of the serological tests which detect agglutinating antibodies are twofold. First, in their failure to detect antibodies in the 9/104 milk samples which were MRT, CF and culture positive, and secondly, in their failure to distinguish between a natural brucella infection and adult vaccination. In an animal vaccinated more than 6 months before testing, a positive whey CF test is indicative of a brucella infection, irrespective of whether it is lactoglobulin or a serum globulin that is detected. CF antibody disappears from the blood within 6 months of adult vaccination (W. R. Kerr, pers. comm.).

We have found the whey CF test to be useful for the detection of infected animals within the herds of producer-retailers in the north of Lancashire and believe it could be a valuable additional test in an eradication programme.

SUMMARY

Two hundred and ninety-three milk ring test-positive individual milk samples were examined by whey complement fixation and agglutination tests and the results were compared with the isolation of *B. abortus* by cultures made from the milk.

There was a closer correlation between whey CF tests and isolation of the organism than between whey AG tests and isolation. Brucella organisms were not isolated from 116 MRT-positive milk samples when the whey CF titre was < 1/10; whereas 9/104 (9 %) isolations were made from milk when the whey AG titre was < 1/20.

Because of its closer correlation with culture results it is suggested that the whey CF test would be valuable in an eradication programme for the detection of infected udders.

The limitations of the milk ring test for the examination of individual milk samples are emphasized and it should be used only as a preliminary screening test. In the individual milk samples examined, 32 % of the MRT-positives showed no evidence of infection by either the whey AG, whey CF, or culture tests.

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