J. Hyg., Camb. (1960), **58**, 473
Printed in Great Britain

Brucellosis in dairy herds—some applications of the milk ring test

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(*Received* 18 *July* 1960)

INTRODUCTION

Brucellosis continues to attract attention in many countries including Great Britain, partly on account of its agricultural implications but also because of its definite, if somewhat unmeasured, importance as a public health problem (Dalrymple-Champneys, 1960; Wallis, 1959). In this latter connexion, Ross (1958) considers that most cases of undulant fever in England are not due to the ingestion of infected milk, but to contact with infected material from occupational or other circumstances. However, others take the opposite view (Dalrymple-Champneys, 1960; Davies, 1957; Wallis, 1957, 1958, 1959; and Bothwell, 1960) and consider milk to be an important source of brucella infection in the human cases they have encountered.

Work on bovine brucellosis in Britain in recent years has covered several different aspects of the problem. Surveys of the incidence of brucellosis in cattle have been carried out by a working party of the Public Health Laboratory Service (Report, 1956) which reported an incidence of *Brucella abortus* in 22,000 milk samples of between 9·93 and 11·58 %, and by Kerr, Pearson & Rankin (1958) in Northern Ireland. In the south-west of England, Wilson (1959) has reported that *Br. abortus* is still an important cause of abortion in cattle. Marr & Williams (1958) also reported an appreciable incidence of brucella infection of dairy herds in northern Scotland.

Many workers have studied the various diagnostic aids available, such as the vaginal mucus test, whey agglutination tests and the milk ring and related tests (Kerr *et al.* 1958, 1959; Marr & Williams, 1958; Jameson, 1957; Report, 1956; Ogonowski & McDiarmid, 1954; and McDiarmid, Findlay, Jameson, Phease, Walker, Jones & Ogonowski, 1958). In most cases those workers have assessed the value of the milk ring test (MRT) and other tests as screening procedures and as means of identifying herds infected with *Br. abortus*. Comparatively little work has been done on its use in the detection of individual infected cows in herds known, or suspected, to be infected. The importance of this aspect to Local Authority Medical Officers was discussed in a series of contributions to the *British Medical Journal* (Any Questions, 1958; Milne, 1958; Ross, 1958; Wallis, 1958).

This paper is concerned with three aspects of the brucellosis problem. In the first place an account is given of the procedure developed and adopted in the course of herd investigations where a dairy herd is known, or suspected, to be

producing brucella-infected milk. Secondly, following observations in an earlier paper (Ferguson & Robertson, 1954) on the possible effect of strain 19 vaccination of adult cows on the MRT reaction of herd milk samples, an experiment is reported in which adult cows were vaccinated with strain 19 vaccine and the MRT reactions of their milk studied. Lastly, the paper reports the result of a survey of routine herd milk samples taken in southern Scotland and examined biologically and by means of the MRT.

MATERIAL AND PROCEDURE

The herd investigational work was carried out in co-operation with Local Authorities either as a result of reported human cases of brucellosis, or as a result of the finding of *Br. abortus* in routine herd bulk milk samples. The material available was usually bulk or quarter milk samples from individual cows in the herds concerned; but where possible, strippings were taken after the cow had been milked. There were few opportunities of obtaining blood or vaginal mucus samples.

The milk samples were subjected to the MRT using Weybridge antigen. In furtherance of the observations made in earlier papers (Ferguson & Robertson, 1954; Holm, Eveleth & Rheault, 1950; and Drimmelen, 1951) on the effect of dilution on the interpretation of the MRT, all samples positive on direct testing and all abnormal samples were retested using a dilution technique. Where the dilution technique was used in the MRT, a negative milk with good fat content from a herd free of brucellosis was used and doubling dilutions were made starting at 1/5. Cream and sediment from the samples were cultured on 5 % serum glucose agar containing gentian violet, 1/25,000, and malachite green, 1/50,000, and incubated in air and in air plus 10 % CO_2. Colonies of brucella were identified first by their appearance and then confirmed by agglutination tests with a known positive serum.

Individual and bulk samples were also inoculated into guinea-pigs which were killed after 6 weeks. Agglutination tests (Weybridge Method I) were carried out on the guinea-pig sera and the spleens were cultured, after maceration, in the same manner as the milk samples.

The effects of strain 19 vaccination on reaction to the MRT were studied by vaccinating fifteen cows all previously free from clinical brucellosis and negative, over a period of 3–5 years, to the blood-serum agglutination and MRT's. The cows came from three different herds. Blood and milk samples were examined over periods ranging from 6 months to 1 year. In the case of milk samples positive on direct testing a series of dilutions were also examined.

The survey work is the result of the examination of sera and spleens from guinea-pigs which had been inoculated with material prepared from routine samples taken by Local Authorities in many areas of southern Scotland. The milk samples themselves were also subjected to the MRT but were frequently 2 days old when received by the laboratory.

RESULTS

Herd investigations

(a) Observations on infected cows

At an early stage in the investigations it was possible to examine over a period of about 40 days a cow shown to be infected with *Br. abortus*. *Br. abortus* was recovered regularly from the milk both by cultural and biological methods from each quarter of the udder. Throughout the period of observation the blood serum showed + + agglutination with *Br. abortus* antigen (Weybridge) at dilutions varying from 1/1280 to 1/2560.

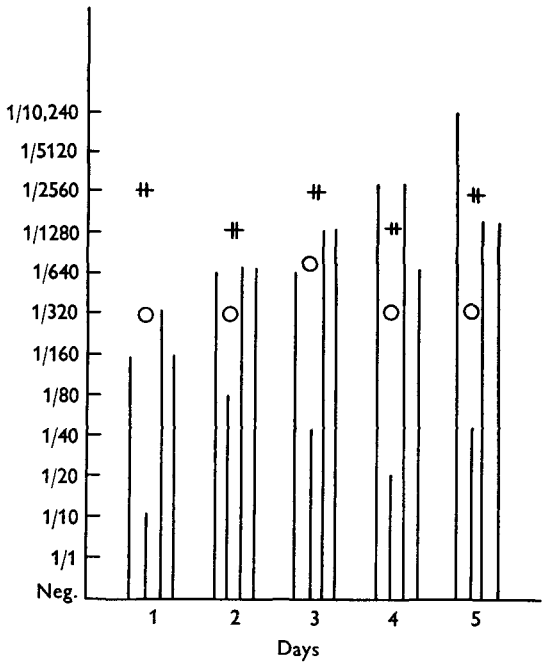

Fig. 1. Cow with proved brucella infection of the udder. Bulk and quarter MRT titres and blood serum titre on successive days. Vertical lines indicate dilution at which quarter samples gave a + + or + + + MRT reaction, the quarters are represented from left to right, LF, RF, LH, RH, on each day. ○ indicates the dilution at which bulked milk from the infected udder gave a + + or + + + MRT reaction. ⧺ indicates dilution at which the blood serum showed 50 % agglutination.

Milk ring tests carried out on bulked quarter samples gave a positive reaction at dilutions ranging from 1/320 to 1/2560 throughout the period. The examination of quarter samples by the dilution method gave interesting results in that there was a considerable variation in the titre of the different quarters. Representative findings are shown in Fig. 1. Further observations on six other infected cows confirmed these findings, namely, that in an infected cow the MRT titre of the bulk milk remains at + + or + + + on dilution to at least 1/10 and that the quarters yield milks of varying MRT titres. It was consequently decided to study

these observations further. This was done as and when herd investigations were carried out and is described in the following section.

(*b*) *Detection of individual infected cows in a herd*

The procedure adopted on receipt of information that a herd was producing brucella-infected milk was as follows. After milking, strippings were obtained from all the cows in the herd together with information regarding mastitis, stage of lactation and yield. The individual composite samples were ring tested immediately on arrival in the laboratory or after overnight refrigeration at 4° C. All samples giving a + + or + + + reaction, as well as abnormal samples, were retested after dilution. The milk samples ring tested using the dilution technique fell into two groups:

(i) those proving negative, ± or + at 1/10, and

(ii) those continuing to give a good positive reaction (+ + or + + +) at 1/10.

Those in the latter group were considered to be probably from infected animals and those in the former, for the purpose of this work, as suspicious. All milk samples from individual cows were cultured using the technique described earlier.

Table 1. *Examination of* 384 *individual cows in herds known to be producing brucella-infected milk*

MRT reaction					
		+ + or + + + direct			
Neg. and ±	+	Neg. 1/10	+ 1/10	+ + or + + + 1/10 or >	Shown infected by culture and/or biological examination
290	.	.	.	.	Nil
.	16	.	.	.	Nil
.	.	32	.	.	1
.	.	.	5	.	Nil
.	.	.	.	41	35

Biological examinations were carried out in the following manner:

Individual samples—milk samples which gave + + or + + + MRT reaction when diluted to 1/10.

Bulk samples, the milk from up to four cows—cows giving a positive (+ + +, + + or +) MRT reaction on direct testing but −, ± or + at 1/10 dilution.

Bulk samples, the milk from up to ten cows—all MRT negative milks, and in separate groups, those milks reading ± on direct testing but − at 1/10.

Further, when composite samples diluted to 1/10 continued to give a positive MRT reading, separate quarter samples were obtained. These were ring-tested by the dilution methods and were also cultured and inoculated into guinea-pigs.

The results of the examination of some 384 cows in twelve herds using the MRT on composite samples from individual cows are summarized in Table 1. It will be seen that of 41 samples giving a + + or + + + MRT reaction direct and when diluted to 1/10 or more 35 (85 %) were shown to be infected. In only one case was

infection proved where the milk, though positive to the direct MRT, was negative when diluted to 1/10. Not one of more than 300 individual cow samples giving a negative ± or + MRT reaction was shown to be infected in spite of extensive cultural and biological examinations.

Table 2 shows in detail the results of the examination of forty-six cows whose milk gave a + + or + + + MRT reaction at 1/10. Here again it will be seen that

Table 2. *MRT titre and brucella infection in milk samples giving a positive reaction* (+ + *or* + + +) *at* 1/10 *or more*

MRT reaction + + or + + + at a dilution of	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Total
Number	10	12	14	4	3	1	2	46
Shown infected by culture or biological test	6	10	13	4	3	1	2	39
Not shown to be infected	4*	2	1	0	0	0	0	7

* One cow newly calved at time of testing, one cow suffering from mastitis.

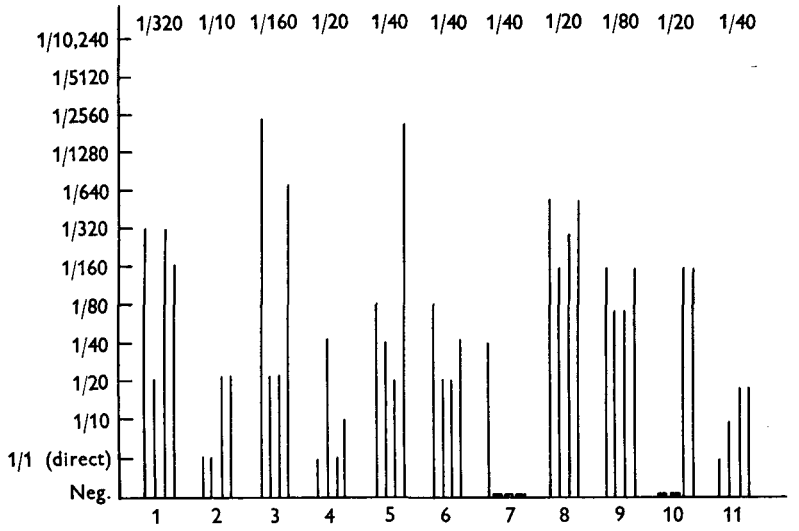

Fig. 2. Cows with proved brucella infection of the udder. Bulk and quarter MRT titres. Vertical lines indicate maximum dilutions at which quarter samples gave a + + or + + + MRT reaction. ▬ indicates a +, ± or −MRT reaction on direct testing. Numbers shown across the top of the figure indicate maximum dilutions at which the bulk milk of each cow gave a + + or + + + MRT reaction. Numbers below the base line indicate individual cows.

almost 85% of samples giving a + + or + + + MRT reaction at a dilution of 1/10 or more were shown to be infected.

The variation in the MRT titre of milk from the different quarters of infected cows (see Fig. 1) was further investigated and representative results are shown in Fig. 2. These confirm the findings that in infected cows composite samples give a positive MRT reaction at dilutions of 1/10 or more and quarter samples vary considerably in MRT titre.

https://doi.org/10.1017/S0022172400038602 Published online by Cambridge University Press

Observations on adult cows vaccinated with strain 19 *vaccine*

The typical reactions to strain 19 vaccination of previously negative cows in milk are shown in Table 3. The numbers of cows sampled at any one time show variation as some became dry and others were disposed of during the period of observation.

A positive MRT reaction usually followed some 8–10 days after vaccination and persisted in some cases for over a year. Blood serum agglutinins appeared about the fifth day, reached a maximum titre of 1/1280 by the thirtieth day and declined to between 1/40 and 1/80 by about the seventieth day. At this level they persisted throughout the period of observation.

Table 3. *Bulk MRT reactions of strain* 19 *vaccinated cows*

Nature of MRT reaction	Days of experiment: Pre-vacc.	0	5	10	15	20	30	40	60	100	180	360
	Numbers of cows											
+ + +, + +, or +, direct and diluted to 1/10 or more	0	0	0	3	5	0	1*	0	2†	1†	0	0
+ + +, + + or +, direct only	0	0	1	6	8	13	7	8	6	6	5	3
Neg. (incl. ±)	15	15	12	4	0	0	5	4	6	8	3	2
Total no. of cows	15	15	13	13	13	13	13	12	14	15	8	5

* Going dry. † Newly calved.

Where cows were in milk at the time of vaccination the intensity of the MRT reaction appearing after 8 days varied from + + + on direct testing only to + + at 1/10 dilution in about 30% of the cases. In all cases, however, after 30 days positive MRT reactions were seen on direct testing only until the 'drying-off' phase of lactation was reached.

In lactations subsequent to the ones in which vaccinations occurred, MRT reactions were variable; in some cases the milk was MRT negative 360 days after vaccination, in others it remained positive.

When a cow was dry at the time of vaccination positive MRT reactions at 1/10 dilution were observed during the first few days of the subsequent lactation. Positive MRT reactions on direct testing only persisted for up to 50 days after calving when they declined, reappearing as the end of lactation was reached.

With respect to quarter samples from individual cows relatively little variation in MRT titre between quarters was seen throughout the experiment. Where a difference was seen, it was merely in the intensity of reaction at a given dilution that is, within the range + to + + +.

Where abnormal secretions were examined, for example, on the day of calving, or when the udder secretion was quite serous at the end of lactation, very high titres were seen in some cases, but in these also the quarter samples from any one cow showed reactions which were similar.

Incidence of Brucella abortus *in herd milk samples*

The investigations reported here are a continuation of the earlier series reported by Ferguson & Robertson (1954) but include a number of areas not previously investigated. The results shown in Tables 4 and 4*a* indicate that in the Midlothian series about 9 % of the milk samples contained *Br. abortus*. Table 5 shows that, under the conditions of sampling, the incidence of brucella infection of milk samples from south-east Scotland, excluding Midlothian, and west and south-west Scotland is lower, viz. 5·3 and 3·5 %, respectively.

Table 4. *Midlothian I* (1953/54)

	Milk ring test reaction				
	+ + and + + +	+	±	−	Totals
Biol. pos.	10	2	0	2	14
Biol. neg.	56	16	5	55	132
	66	18	5	57	146

Table 4*a*. *Midlothian II* (1955/59)

	Milk ring test reaction				
	+ + and + + +	+	±	−	Totals
Biol. pos.	19	0	0	3	22
Biol. neg.	45	18	11	146	220
	64	18	11	149	242

Table 5

	Samples examined	Ring test pos. (+ + or + + +)	Ring test pos., Biol. pos.	Biol. pos., Ring test neg.
S.E. Scotland excluding Midlothian	113	21	6	0
W. and S.W. Scotland	400	96	14	0

DISCUSSION

Detection of cows with brucella udder infections

The observations reported indicate that the MRT, when used as described in this paper, has considerable value for the detection of infected cows in a dairy herd and in the differentiation of such cows from those whose milk is positive to the MRT merely as a result of strain 19 vaccination.

In forty-six out of a total of forty-seven cases of proved udder involvement two phenomena were constantly exhibited. These phenomena were that the milk from an infected cow will continue to give a positive MRT reaction at a 1/10 dilution or more, and that quarter samples will also show relatively high titres but with considerable variation from quarter to quarter. One cow only out of the forty-seven was exceptional in that although its milk was MRT negative when diluted

1/10, udder infection was proved. Unfortunately, there was no further opportunity of resampling this animal.

In the practical application of these criteria certain reservations must be borne in mind, namely:

(i) Recently calved cows, i.e. up to 30 days after calving, although neither infected nor vaccinated may produce milk with a relatively high MRT titre. Such cows will, however, show practically uniform titres in quarter samples.

(ii) Cows giving less than 10 lb./day, i.e. going dry or practically dry, will give similar results.

(iii) Cows with mastitis present something of a problem, as of course, mastitis and brucella infection may be present together. As would be expected the abnormal mastitis secretion has a high antibody content and on dilution may give a high MRT titre if brucella antibodies are present. Such 'milks' are, however, obviously abnormal and give unsatisfactory results on direct testing. These unsatisfactory results include failure to develop a cream ring either white or coloured, decolorizing of the antigen and the formation of granular deposits. However, even though mastitis may be the sole cause of a high MRT titre in a quarter sample there is no way of proving this, and cows yielding such samples must be regarded as possibly infected with brucella until shown to be clear by culture and biological examination, or until the mastitis resolves and more satisfactory MRT results are obtained. In this series the few cases of subclinical mastitis encountered did not interfere with the procedure outlined above. That is, milk samples judged to be abnormal using the Whiteside test (Murphy & Hanson, 1941), even though they gave a strong positive MRT reaction on direct testing, generally did not give a positive reaction at 1/10 and quarter samples showed an even titre. Only one instance occurred in which the bulk milk gave a positive MRT reaction at 1/10 and also displayed uneven MRT titres in quarter samples, but brucella infection was not proved in this case. Thus whilst it has to be recognized that various forms of mastitis may interfere with the interpretation of the MRT technique described above, this does not seriously affect the usefulness of the procedure. However, it would be necessary to regard as suspect all cows whose milk gave a positive MRT reaction at 1/10 even though there is evidence of mastitis.

(iv) Newly vaccinated cows in milk may show a positive MRT reaction at 1/10 for up to 30 days after vaccination, but quarter MRT titres will be uniform. After 30 days positive MRT reactions will not be seen at dilutions of 1/5 or more until the cow becomes dry again. Cows dry at the time of vaccination may also show a positive MRT reaction during the early stages of the subsequent lactation.

Cows falling in the above categories must, therefore, be regarded as suspect until on resampling they fall within a different category or are shown to be infected.

The use of the procedure described has resulted in a speeding up of herd investigations where brucella infection of the milk is suspected, allowing early detection of the cows most likely to be infected. In these circumstances diagnostic aids such as cultural and biological examination can be intensively applied to the

cows most likely eventually to prove infected. Other cows in the herd can, on the evidence of the work reported here, be regarded as free of udder infection. It is not suggested that the procedure would have quite as high a value for eradication purposes, as cases would undoubtedly be met with where there was no udder involvement.

These findings tend to support the claim of Drimmelen (1951) that the MRT can be used to distinguish between vaccinated and infected cows though his actual findings have not been confirmed. Drimmelen reported that no positive MRT reactions were encountered in cows vaccinated more than 4 months previously; in this work vaccinated cows continued to give positive MRT reactions on direct testing though not when the dilution techique was used.

Neither are the observations in complete accord with those of Marr & Williams (1958) who reported negative MRT results in eight cows 6 weeks after vaccination. It is, therefore, not possible to agree entirely with these workers that 'strain 19 vaccination even in adults does not necessarily interfere significantly with the result of a MRT made on the bulk'. It is clear that the effect of adult vaccination in a dairy herd on the bulk MRT of that herd will depend upon the number of cows vaccinated at any one time and on the time of sampling in relation to the time of vaccination.

Kerr *et al.* (1958) using the milk whey agglutination test also reported on the difference between vaccinated and infected cows. They too, found cows whey negative but biologically positive, nevertheless, 99 % of their biologically positive milks showed a whey titre of 1/10 or more. In their experimentally vaccinated animals all whey agglutination tests were negative 12 weeks after vaccination and at no time exceeded 1/20 although serum titres ranged from 1/160 to 1/1280.

McDiarmid *et al.* (1958) studying experimental cattle have shown that in the examination of eighteen cows with confirmed brucella infection of the mammary gland, the MRT was negative in 5 % of the samples examined. In the work reported here none of the 290 individual cows giving a negative MRT reaction and none of the sixteen giving a + reaction were shown to be infected. On the other hand, of seventy-eight samples giving a + + or + + + reaction on direct testing, proof of infection was obtained in only thirty-six cases, but thirty-five of these were from forty-one cows whose bulk milk samples gave a positive MRT reaction at 1/10 dilutions.

An explanation of the phenomena described above may revolve round the question of local production of antibodies in the udder. Kerr *et al.* (1958, 1959) and Porterfield, Petersen & Campbell (1959) have reported on the production of antibodies by the bovine udder. It is clear that infusion of the udder with various bacterial antigens will stimulate the production of specific antibodies within the gland. In earlier work, Mitchell & Duthie (1930) showed that removal of the udders of cows infected with *Br. abortus* resulted in a decline in antibody production. Kerr *et al.* also state that serum agglutinins do not appear in the milk except under certain recognized circumstances, namely, in milk of early lactation, late lactation and in some forms of mastitis.

Using the milk whey test to study milk agglutinins, Traum & Maderious (1947)

reported that agglutinins in low levels were found in the milk of vaccinated cows for periods up to 90 days after strain 19 vaccination, and that as strain 19 was not recovered from over 5000 milk samples from vaccinated cows, they considered it was probable that these milk agglutinins resulted from diffusion from the blood stream rather than from agglutinogenesis within the udder. Similarly Cameron, Kendrick & Merriman (1956) and Cameron & Kendrick (1957) claimed that whey reactions became negative within 3 months after vaccination in non-infected animals and that the whey test could, therefore, be effectively used to differentiate post-vaccination reactions from these caused by virulent infection.

It seems reasonable, therefore, to deduce from the observations here reported, and from the findings of other workers referred to, that cows suffering from a brucella infection of the udder will behave differently from these merely vaccinated with strain 19 vaccine. In the case of udder involvement a relatively high level of milk agglutinins and a variation in their level from quarter to quarter would be expected. It would, however, require extensive work of a bacteriological and histological nature to relate actual infection of a quarter to the agglutinin content of the milk of that quarter. This, it has not been possible to do in the present work. In the case of strain 19-vaccinated cows it would appear that the milk agglutinins to brucella antigens are derived from the blood serum; they will, therefore, not reach high levels except when abnormal udder conditions are present, nor are they likely to be very different in level from one quarter to another.

Since this work was commenced improvements in media used for the isolation of *Br. abortus* have been made (Mair, 1955; W.H.O., 1958; Jones & Morgan, 1958). In future the medium used in this work will be replaced by the 'serum-dextrose-agar with antibiotics' described by Jones & Morgan (1958). Such a medium appears to suppress the growth of contaminants without seriously inhibiting certain strains of brucella.

Brucella-infected milks

Since the previous survey by Ferguson & Robertson (1954) few figures have been published to show the incidence of *Br. abortus* in milk. However, in a Report (1956) the Public Health Laboratory Service Working Party recorded an incidence of *Br. abortus* of between 9·93 and 11·58 % in more than 22,000 milk samples.

Human cases of brucellosis continue to occur and a large proportion of these may well have resulted from the drinking of infected milk. In spite of extensive pasteurization of the national milk supply it seems that there are still frequent opportunities for the ingestion of untreated milk. These opportunities are probably not confined to rural and small urban areas as untreated milk, though relatively small in volume, is probably widely distributed, many households taking in both pasteurized and untreated milk. In the Edinburgh area the figure of about 9 % infected samples (Tables 4, 4*a*) shows little change from that of about 10·5 % reported by Ferguson & Robertson (1954).

In this survey the MRT failed on five occasions in 388 (1·2 %) to detect herd samples subsequently shown to be infected. On the other hand, of 166 samples giving a +, + + or + + + MRT reaction, thirty-one were shown to be infected

(Tables 4, 4*a*). The proportion of herd milks giving a positive reaction (+ + or + + +) to the MRT, (43 %), is somewhat lower than in the previous series (53 %) and higher than the 25 % found by Marr & Williams (1958) though they also regarded ± reactions to the MRT as positive. In Table 5 dealing with other areas where the recovery rate of *Br. abortus* was lower (5·3 and 3·5 %) the MRT did not fail to detect samples which were ultimately proved to be infected.

Thus the opinion of other workers that the MRT is useful as a screening test for herd milk samples is confirmed. The failure of the MRT to detect a small percentage of samples which were proved biologically to contain *Br. abortus* indicates the need to consider the dilution factor when single bulk samples from large herds are examined. It is also necessary to retest herds periodically as infection of the udder may be present before milk agglutinins appear.

SUMMARY

1. It has been shown that cows infected with brucellosis of the udder can usually be detected by an application of a modified milk ring test, based on the finding that milks from infected cows, in addition to showing a high bulk MRT titre also show considerable quarter variations. The effects of adult vaccination with strain 19 vaccine on the MRT are reported; low bulk titres with uniformity between quarters, except at the beginning and end of lactation and immediately following vaccinations, were observed, thus making differentiation between vaccinated and infected cows possible.

2. In a survey of raw milk samples from three areas in southern Scotland, 3·5, 5·3 and 9·0 % of samples were shown to be infected with *Br. abortus*.

Thanks are due to Dr Stableforth, Director, M.A.F.F. Veterinary Laboratory, Weybridge, for providing brucella antigens and to the Bacteriological Departments of Edinburgh University, and the Astley Ainslie Hospital, Edinburgh, and to the Bacteriological Laboratory, Corporation of Glasgow for providing post-mortem material from guinea-pigs as well as milk samples and relevant information.

Thanks are also due to the Bacteriological Department, Royal (Dick) School of Veterinary Studies for co-operation, particularly in the biological examination of milk samples.

The co-operation of many Local Authority officers and the assistance of Mrs J. Stewart in the laboratory work is gratefully acknowledged.

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