Arch. Inst. Razi, 1972, 24, 103-111

COMPARISON OF VARIOUS SEROLOGICAL TESTS ON THE MILK OF SHEEP IN RELATION TO THE ISOLATION OF *BRUCELLA MELITENSIS*

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SUMMARY

A total of 173 milk ring test (MRT) positive and 356 MRT-negative individuals from 2735 sheep in various stages of lactation were tested by whey complement fixation test (CFT), whey Coombs' test or antiglobulin test (AGT) and whey agglutination test (AT). These findings were compared with the rate of isolation of *Brucella* organisms obtained on culture. The whey CFT and whey AGT were more specific and showed a closer relationship to the excretion status of *Brucella* than whey AT or MRT.

Out of 2735 samples of milk 68 MRT-positive and 6 MRT-negative milk samples from aborting sheep were found to be positive on culture. Out of 173 MRT-positive milk samples 58 (33.5 per cent) proved negative culturally and also gave negative results to the other tests.

The MRT is less specific than other serological tests for identifying sheep excreting *Brucella*. However, it is of value as an initial screening test for *Brucella* infection in milk.

INTRODUCTION

Brucella carriers may be found in naturally infected flocks of sheep and goats. These may not be identified as infected animals since they have normal parturitions and they remain in the flocks as sources of infection for other animals or humans. Identification of carrier animals is therefore most important.

The serological tests most widely used to identify *Brucella* infected animals

Reprinted from British veterinary Journal volume 127, p. 105, (1971).

are the blood serum agglutination test (SAT), the milk ring test (MRT) and the whey agglutination test (AT, tube and plate methods).

In recent years, the complement fixation, Coombs' and other tests have been used as supplemental methods on blood serum and whey of various species. Milk is easier to obtain than blood and, as infected animals may excrete organisms, it can be used for culture.

This paper reports on the comparative value of some serological tests on milk from sheep which were screened by the milk ring test. Cultures and serology were performed on milk reacting to the ring test and from sheep which had aborted. Excretion of *Brucella melitensis* in milk confirmed by culture was considered to be the criterion of infection.

No references were found on the use of the whey CF or whey Coombs' tests (AGT) in sheep or goats' milk.

MATERIALS AND METHODS

Selection of sheep

Studies were conducted on 3500 unvaccinated native fat-tailed sheep on a state-owned farm. Nine separate flocks of approximately equal size were examined but each had contact with the others. Milk from sheep in various stages of lactation and from aborting sheep was studied. The normal lambing period was from early March to the beginning of May. Specimens were collected during a five-week period in April and May 1969. The milk ring test was performed on samples from 2735 sheep. Cultures were made from the milk of those reacting to this screen test, from 356 MRT-negative specimens and from thirteen aborting sheep. All milk specimens cultured were examined serologically by the whey tube agglutination, whey complement fixation, and whey Coombs' tests. The clinical histories varied in each flock. The remaining 765 sheep had to be omitted for many reasons, such as being dry following a normal lactation or an abortion, infertility and immaturity.

Serology

Whey agglutination test (AT). Whey was prepared by adding 1-2 drops of rennet to 5-6 ml of defatted milk obtained by centrifugation. The tubes were allowed to incubate at 37°c for 1 hour. After a second centrifugation, whey was tested using the blood serum tube agglutination method except that 5 per cent saline was used as diluent in order to minimize the prozone phenomenon. A 50 per cent agglutination or more in the 1: 10 (20 I.U.) or higher serum-antigen dilution was considered a positive reaction.

Whey complement fixation test (*CFT*). This was performed according to the Kolmer technique with cold fixation as described by Alton & Jones (1967). Veronal buffer was used as a diluent. Whey was inactivated at 60°c for 30 minutes. Strain 99 *Brucella abortus* antigen was diluted 1: 100 in normal saline. Whey samples giving at least 50 per cent inhibition of haemolysis at a dilution of 1: 5 or higher were considered positive for infection.

Whey Coombs' or antiglobulin test (*AGT*). The methods of performing this test have been described by Hadju (1963). To 0.2 ml of whey was added o.8 ml of saline and these were incubated in a water bath at 70°c for 10 minutes. Serial dilutions were made to give final dilutions of 1: 10, 1: 20, etc., until end-point titres were reached. To each tube was added an equal amount (0.5 ml) of Strain 99 antigen, mixed and incubated at 37°c for 2 hours. Then 1.5 ml of saline was added to each tube and the cells deposited by centrifuging at 3000 r.p.m. for 20 minutes. The supernatant was discarded and the deposit washed three times in saline. After the final washing, 1 ml of rabbit anti-sheep globulin serum diluted to optimal titre was added to the tubes and the deposit resuspended by shaking. The tubes were incubated overnight. Agglutination of 50 per cent or greater in the 1: 10 or higher dilutions was considered positive. Optimum titre of rabbit anti-sheep globulin serum was found by titration against known *Brucella*-positive sera. This was the highest dilution which gave correct titre of the *Brucella*-positive sera.

Milk ring test (*MRT*). To 1 ml of a milk sample in a narrow tube (7 mm) was added one drop of haematoxylin-stained antigen prepared at the Razi Institute. These were gently mixed and incubated at 37°c. The test was read and results recorded after 3 hours of incubation.

Antigen. All antigen used for serological tests was that prepared from *Brucella abortus* 99 at the Razi Institute and standardized against the International Standard Anti-*Brucella* serum so as to give 50 per cent agglutination with a final dilution of 1: 500 of the serum-antigen mixture. Fifty per cent agglutination at serum dilutions of 1: 10, 1: 20 and 1: 40 therefore represent 20, 40 and 80 international units (I.U.) respectively (WHO Report, 1958). The antigen used for the CF test was standardized to give 50 per cent haemolysis at a dilution of 1/100 of the International Standard Anti-*Br. abortus* serum, using overnight fixation at 40°c.

Bacteriology. Individual milk specimens of approximately 30 ml were centrifuged at 5000 *g*/30 min (MSE Major). The cream and deposit were mixed and inoculated on serum dextrose agar with antibiotics and ethyl violet (Jones & Morgan, 1958). Each sample was inoculated on 3-5 plates and incubated for 3-6 days at 37°c.

RESULTS

A comparision of the milk ring, whey agglutination, whey complement-fixation and whey antiglobulin tests with results of culture on milk specimens which were MRT-positive is shown in Table I. *Brucella melitensis* was isolated from 68 of the 173 MRT-positive milk specimens (39.3 per cent).

The whey AT was superior to all other serological tests in the percentage of *Brucella* recoveries from positive samples. However, this procedure failed to detect 35 milk excretors found among 123 sheep (28.4 per cent) which were negative to the test. As 14 of 24 whey specimens with titres of 1: 10 (20 I.U.) were culturally positive, a more critical criterion of classification may be necessary

An evaluation of the results of the whey CFT showed that the sensitivity was equal to that of the whey AT. Of the 88 whey CFT-positive specimens 58 (65.9 per cent) yielded *Brucella.* The test specificity was superior to all others and only 10 of the 85 (11.7 per cent) whey CFT-negative samples were positive on culture.

The whey AGT properly identified 58 excretors in 100 (58 per cent) positive samples and failed to identify 10 shedders in 73 (13.6 per cent) serologically negative samples. Thus, the sensitivity proved to be slightly inferior to that of the whey CFT but superior to that of the MRT or of whey AT, and the specificity was slightly inferior to that of the whey CFT but far superior to that of the whey AT.

As expected, the correlation of *Brucella* recoveries to titres is better in those positive in higher dilutions, but low titre reactions in all serological procedures proved to be significant. With the criteria used for classification, 14 of 24 (58.3 per cent) in the whey AT, 16 of 32 (50 per cent) in the whey CFT, and 9 of 24 (37.3 per cent) in the whey AGT were culturally positive in the lowest dilution in which a reaction was considered important.

	Serologic Test and Titre																												
	MRT			*Whey AT*							*Whey CFT*								*Whey AGT*										
	+	++	+++	<1:10	1:10	1:20	1:40	1:80	1:160	1:320	<1:5	1:5	1:10	1:20	1:40	1:80	1:160	1:320	<1:10	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Number examined	52	38	83	123	24	13	6	4	2	1	85	32	20	22	7	5	1	1	73	24	16	22	23	7	4	3	1		
Number culturally positive	13	20	35	35	14	9	4	3	2	1	10	16	17	12	7	4	1	1	10	8	6	16	15	5	4	3	1		
No. culturally positive/ No. serologically positive Percentage	68/173 (39·3)			33/50 (66·0)							58/88 (65·9)								58/100 (58·0)										
No. culturally positive/ No. serologically negative Percentage				35/123 (28·4)							10/85 (11·7)								10/73 (13·6)										

\+ = cream ring same colour as milk column.

++ = cream ring darker than lower milk column.

+++ = well-defined cream ring on top of white milk column.

The correlation between the MRT reactions and the degree of excretion of *Brucella* is shown in Table II.

TABLE II

CORRELATION OF RING TEST REACTION AND EXCRETION OF *Brucella* IN SIXTY-EIGHT MRT-POSITIVE SHEEP EXCRETING *Brucella melitensis*

Degree of MRT	a+		a++		a+++	
Culture positive	13		20		35	
Percentage of total	19·1		29·4		51·5	
	No. of sheep	*No. of colonies*	*No. of sheep*	*No. of colonies*	*No. of sheep*	*No. of colonies*
Degree of excretion correlated with MRT category	7	+	11	+	4	+
	1	++	2	++	13	++
	2	+++	2	+++	12	+++
	3	++++	5	++++	6	++++

Degree of excretion correlated with MRT category:
+ = 1–9 colonies
++ = 9–99 colonies
+++ = 99–999 colonies
++++ = > 999 colonies

The degree of MRT:
a+ = cream ring same colour as milk column
a++ = cream ring darker than lower milk column
a+++ = well-defined cream ring on top of white milk column

An analysis of these results and comparison with those in Table I indicates that the strength of MRT reaction has little relation to infection rate or degree of excretion. Of the 68 shedders, 19.1 per cent had a 1+reaction to the MRT. Of the numbers of milk samples examined, 13 of 52 (25 per cent) with 1+,20 of 38 (52.6 per cent) with 2+,and 35 of 83 (42.2 per cent) with 3+reactions on the MRT were culturally positive.

No evidence of infection was found by the other procedures in 58 (33.5 per cent) of the 173 MRT-positive milk samples examined. This suggests that use of the MRT as a sole criterion for detecting individual infected sheep is unreliable.

There were only 13 sheep that aborted from the group of 173 that had MRT-positive milk. Of these 13 aborted sheep, 9 were positive on culture and other serological tests.

At a later stage it was decided similarly to investigate the position in 356 MRT-negative milk samples to act as a control group. Six of those from sheep with a history of abortion were found to be positive on culture, whey AT, whey CFT and whey AGT serological tests.

All cultures were identified as *Brucella melitensis* biotype I by sensitivity to dyes, failure to produce H2S, monospecific serum agglutination and lack of phage sensitivity.

DISCUSSION

The results reported in these investigations support the work of others on the importance of milk serology for the diagnosis of brucellosis in sheep and goats. Milk from lactating animals is generally easier to obtain than blood or vaginal specimens and in addition to serology, it can be used for cultural examinations.

The MRT is the most widely used procedure on milk and is particularly valuable as a screening test on a herd or flock basis and in individual animals.

Isolations of *Brucella* are more probable in MRT-positive specimens. Only six isolations were made from milk which was negative to this test and all were from aborting sheep. Mathur (1967) showed that abortions and a positive MRT test were two very important indicators of brucellosis in a herd or flock.

No single diagnostic test can be depended upon to detect every infected animal and negative tests may be found in known infected animals (Mathur, 1968). Failure of the blood agglutination test in individual animals has been shown earlier by Renoux, Alton & Mahaffey (1956), and Renoux, Sacquet, Velasquez & Castellani (1957).

Workers have reported results of the complement fixation and Coombs' test in blood serum of goats and sheep. Gargani & Guerra (1967) found the CF test to be very specific in blood serum and it remained positive much longer than the agglutination test in infected sheep. Gaumont (1963) found that CF and Coombs' tests were more specific. Unel, Williams & Stableforth (1969) compared three blood serological tests and found greater specificity with the antiglobulin method.

The whey AT was found to be inferior to other tests evaluated (Table 1) in detecting milk shedders. Mathur (1967) found that the whey agglutination test was less useful than the milk ring test and he found six cases where both were negative in culturally positive animals.

The MRT was found to show some probably "false positive" reactions since 2.2 per cent of 2620 samples which were MRT-positive failed to yield *Brucella* or react to other serologic procedures. This was most likely due to non-specific gamma globulin.

The lactation status of many of the sheep under study could not be determined. Farrell & Robertson (1968) suggested that many a++ and a+ reactions in the MRT on individual milk samples in cattle were in fact false positive. In this study, if only the a +++ reactions were considered significant, 33 shedders with lesser reactions would have been missed.

The whey complement fixation test has been compared in cattle with the whey agglutination test (Farrell & Robertson, 1968) and found to be more specific. *Brucella* was not isolated from complement fixation test negative specimens. In another study (Robertson & Farrell, 1968) the whey complement fixation test showed a closer relationship to infection than either the whey agglutination or MRT.

As seen from the results in Table 1, a proportion of the sheep that were found to be positive for serological tests proved negative on culture. It seems likely that there were some sheep that may have been infected but were not excreting the organism in their milk at the time of examination as *Brucella* is known to be excreted intermittently. For this same reason Mathur (1968) recommended re-examination of sheep from infected flocks. The identification and removal of *Brucella* carriers in flocks of sheep would be of great value in preventing the spread of infection to man and to other animals.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr F. Entessar, Head of the Microbiology Department, Razi Institute, for his interest in the work, to Dr A. Ardalan, of the Bacteriology Department, Razi Institute, for guidance and to Dr P. Nicoletti, Epizootiologist, Food and Agriculture Organization Mission in Iran, for help with the manuscript. He is also thankful to Mr B. Kavousi, Technician of Brucellosis Section, Razi Institute, for his technical assistance.

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Comparaison de différents tests sérologiques effectués sur le lait de brebis en vue de la détection de *Brucella melitensis*

(Ebadi)

Résumé. Le lait d'un total de 173 brebis dont le test de l'anneau était positif (MRT) et de 356 dont le test était négatif, provenant de 2735 brebis à différents stades de lactation, a été testé sur le petit lait par la fixation du complément (CFT), le test de Coombs ou le test à l'antiglobuline (AGT) et le test d'agglutination (AT). On a comparé ces résultats avec le taux d'isolement des organismes *Brucella* obtenus après culture. Les tests CFT et AGT sur le petit lait etaient plus spécifiques et avaient un rapport plus étroit avec l'excrètion de *Brucella* que les tests AT ou MRT.

Sur 2735 échantillons de lait, 68 échantillons de lait MRT positifs et 6 MRT négatifs provenant de brebis ayant avorté étaient positifs après culture. Sur 173 échantillons de lait MRT positifs 58 (33,5 pour cent) furent négatifs après culture et donnèrent également des résultats négatifs par tous les autres tests.

Le test MRT est moins spécifique que les autres tests sérologiques pour identifier les brebis qui excrètent *Brucella*. Il est cependant utile comme méthode initiale de dépistage de l'infection du lait par *Brucella*.