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Comparative Evaluation of Microagglutination Test and Serum Agglutination Test as Supplementary Diagnostic Methods for Brucellosis

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The diagnosis of brucellosis in cattle and small ruminants requires the use of more than one serological test. The complement fixation test (CFT), the rose bengal test (RBT), and the serum agglutination test (SAT) are among the most useful tests for routine diagnosis. The microagglutination test (MAT) was developed as a simpler and more efficient test than the SAT. The relative efficacy of this test compared with that of the SAT was evaluated by using brucella-free sheep and goats prior to and after vaccination treatment. The specificities of the MAT and the SAT were 100%. Of the ewes and goats with a vaccination history, one ewe, expectedly a negative responder, had reactions in the MAT, the complement fixation test, and the rose bengal test but not in the SAT, suggesting a lower sensitivity of the SAT in this case. The calculated sensitivities of the MAT and the SAT were 93.9%. The agreement between MAT and SAT results for nonresponders was examined by using sera from unvaccinated lambs and kids (95.2% agreement), unvaccinated ewes and goats (84.4%), and ewes and goats with a vaccination history (43.9%). For the latter group higher levels of agglutination units were observed by the MAT than by the SAT in 51.5% of the samples. In testing sera from positive reactors after vaccination neither method was superior (MAT values were greater than SAT values for 23.5% of the samples, and MAT values were less than SAT values for 21.9% of the samples). Comparison of the methods on the individual sample level revealed a significant correlation between the MAT and the SAT ($r = 0.96 \pm 0.005$; $P < 0.001$). Since the MAT is simpler to perform than the SAT and can potentially be automated, the inclusion of the MAT as a supplementary test in brucellosis control programs is recommended.

The prevalence of brucellosis in Israel is due to infection of sheep and goats with *Brucella melitensis*. Bovine brucellosis caused by *Brucella abortus* has been eradicated for several years, but cattle may potentially be infected with *B. melitensis* (9, 18, 33). A national program for control of the disease, based upon vaccination of young animals with a living vaccine strain (sheep and goats with *B. melitensis* Rev1 and cattle with *B. abortus* S19) and a test and slaughter policy for infected flocks, has been widely implemented in Israel for many years.

As a result of this program, some vaccinated animals might become positive responders in the serological tests, thus hampering diagnosis of the disease. Therefore, only animals after their first gestation are surveyed for brucellosis, since they are not expected to show a detectable vaccination antibody titer. Conventional serological tests, e.g., the serum agglutination test (SAT), the complement fixation test (CFT), and the rose bengal test (RBT), are the standard tests used in the National Brucellosis Laboratory as the diagnostic measures to identify foci of infection.

All three tests are standardized according to international activity units (1). The CFT is usually considered the most reliable test because of its high specificity and sensitivity. Therefore, this test is recommended as a confirmatory test in the diagnosis of the disease (2, 15, 25, 30). The RBT is similar in sensitivity and specificity to the CFT (24) but nevertheless is recommended as a screening test in the field (4, 11, 34). While the CFT and the RBT mostly identify immunoglobulin G1 isotypic antibodies, which are predominant in the later phase of brucellosis or in chronic brucellosis, SAT response is detected in the early phase of the disease when immunoglobulin M antibodies are elicited (7, 12, 19, 25).

Although the SAT is less specific and less sensitive than the CFT and the RBT (2, 27, 34), it is still being used in several countries as a surveillance method (16). Several facts have led to this decision. Firstly, this test is simpler to apply. Secondly, by use of international standard anti-*B. abortus* serum (ISABS) (also known as the Second International Serum) the test can be standardized according to international agglutination units, making its results comparable in all countries. Thirdly, the diagnosis of human brucellosis is still dependent on this test.

A method based upon measurement of agglutinating antibodies by a microplate agglutination test (MAT) has been reported (14, 22, 28, 35). In a survey of bovine herds at the end of an eradication program, the MAT was found to be superior to the SAT in efficacy. Altogether, it was shown a suitable substitute for diagnosis of human, bovine, caprine, and ovine brucellosis (3, 5, 14, 16, 17, 28). The purpose of this study was to evaluate the use of the MAT in the diagnosis of brucellosis in Israel.

MATERIALS AND METHODS

Serum samples. All serum samples were collected from brucellosis-free flocks. Two groups of animals were used according to the following criteria.

(i) Nonresponders. The first group included animals expected to be negative responders in the agglutination test. It consisted of 83 unvaccinated ewes and goats, 52 vaccinated ewes and goats after their first gestation, and 21 ewe lambs and goat kids prior to vaccination. Most of the animals were tested only once. Several others were tested twice, for a total of 215 tests.

(ii) Responders. The second group included animals expected to be respond-

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TABLE 1. Comparative evaluation of the specificities and sensitivities of the MAT and the conventional SAT

Method	Specificity (%) with serum samples from the following animal group[a]:			Sensitivity (%) with serum samples from vaccinated animals (n = 246)[b,c]
	Unvaccinated (n = 135)[c]	Vaccinated (n = 66)[c]	Total tested (n = 215)[c]	
MAT	100 (97.7–100)	98.5 (91.8–99.9)	99.5 (97.4–99.9)	93.9 (90.3–96.7)
SAT	100 (97.7–100)	100 (94.6–100)	100 (98.6–100)	93.9 (90.3–96.7)

[a] Animals were divided into groups according to the following categories. The unvaccinated group consisted of ewe lambs, goat kids, ewes, and goats. The vaccinated group consisted of ewes and goats after their first gestation. Specificity was calculated as indicated in Materials and Methods. In the column for the total tested, data for seven ewes of unknown vaccination status were included. These animals were sampled and tested twice. Data in parentheses represent contingency by 95% confidence intervals.

[b] The vaccinated group consisted of 246 ewe lambs. Sensitivity was calculated as indicated in Materials and Methods. Data in parentheses represent contingency by 95% confidence intervals.

[c] n, number of serum samples tested per experiment.

ers in the agglutination test. It consisted of 246 ewe lambs and goat kids which were vaccinated between 20 and 40 days prior to the experiment.

Serological tests. The CFT, the SAT, and the RBT were performed as described by Alton et al. (1). The antigen used in the SAT was smooth *B. melitensis* biotype 1 strain Huddleson. The antigen was prepared in the laboratory and standardized for agglutination units by using the Second International Serum (ISABS). A national serum adjusted to give an endpoint reading of 50% agglutination at a final serum dilution of 1:650 was used routinely for antigen titration. A response was considered positive in the SAT when 50% agglutination at a serum dilution of 1:40, equal to 60 IU/ml, was achieved (1).

The antigen for the MAT was prepared in a manner similar to that of the macroagglutination test (SAT). However, standardization was carried out in a microplate instead of in large tubes. A working dilution of the antigen in a stained isotonic 0.5% phenol–saline solution which contained 0.02% safranin O (Sigma) was used. A primary serum dilution of 1:2.5 was further diluted in twofold steps in a 0.5% phenol–5% NaCl solution (1). Fifty microliters of both the antigen suspension and the serum sample dilutions per well were mixed together and incubated for 18 h at 37°C. Positive and negative sera were included as controls. To determine agglutination results, standard antigen suspensions simulating 100, 75, 50, and 25% agglutination levels were included. The MAT was performed in 96-well V-shaped microplates (Nunc, Roskilde, Denmark).

Statistical analysis. Contingency tables were formed with the antibody titer data measured by the MAT and the SAT. Case by case associations between the MAT and the SAT were estimated by using the Spearman correlation. For this purpose, agglutination units were changed to arbitrary units of stepwise increments. One unit was considered negative agglutination; 12.5, 15, 17.5, and 20 U were considered agglutination titers between 1:10 (+1) and 1:10 (+4), respectively. Similarly, the other titers received higher values. A paired t test was used to obtain the significance of the difference between the MAT and the SAT on the individual sample level. The difference of the accumulated titer values obtained from all tested serum samples was compared for the MAT and the SAT by general linear model analysis.

Specificity and sensitivity were calculated from the experimental results by using the criteria of true-negative and true-positive responders from the predetermined brucellosis status of the animals.

Specificity was calculated from the equation

$$\text{specificity} = \frac{\text{true-negative responders}}{\text{true-negative} + \text{false-positive responders}} \times 100 \quad (1)$$

and sensitivity was calculated from the equation

$$\text{sensitivity} = \frac{\text{true-positive responders}}{\text{true-positive} + \text{false-negative responders}} \times 100 \quad (2)$$

RESULTS

The specificity and sensitivity of the MAT were evaluated in comparison with those of the conventional SAT. Specificity was determined by calculating the proportion of false-positive responders among animals expected to be nonresponders. Sensitivity was determined by calculating the proportion of negative responders among animals expected to be positive responders. A 1:40 (+2) titer was used as the cutoff point to determine positive reactors.

As can be seen in Table 1, the specificity of the SAT was 100%, irrespective of the vaccination status of the animals. In comparison, the specificity of the MAT varied between 98.5 and 100%, the values for the vaccinated animals and the unvaccinated animals, respectively. In the first group a single ewe of the 66 tested had a reaction in the MAT at a 1:40 (+2) antibody titer, while in the SAT it showed a 1:20 (+4) titer.

The specificities of the two methods were also evaluated for all tested samples combined, i.e., those from unvaccinated animals, those from vaccinated animals, and those from seven animals from which samples were collected twice (Table 1, total tested). The specificity of the MAT was 99.5%, and that of the SAT was 100%. This difference between the methods, as analyzed by normal approximation, was not significant.

Ninety-five percent confidence intervals calculated from a binomial distribution are shown in parentheses. As can be seen, deviation of the results was allowable for 97.7 to 100% of the samples in the unvaccinated group in both the SAT and the MAT. For the vaccinated group the confidence interval was somewhat less stringent in the MAT (91.8 to 99.9%) than in the SAT (94.6 to 100%). This is accounted for by the presence of the above-mentioned positive responder in the MAT.

The sensitivities of both the MAT and the SAT were tested with ewe lambs and goat kids which were vaccinated a few weeks prior to the test. As shown in Table 1, the calculated mean value of sensitivity was 93.9% for the two methods. Confidence intervals were also identical, ranging between 90.3 and 96.7%.

The percents agreement between the two methods are depicted in Table 2. For the animals expected to be nonresponders, the extent of agreement between the SAT and the MAT varied according to the vaccination status of the animals. The best agreement, 95.2%, was observed for group II, which

TABLE 2. Percents agreement between the results of the MAT and the SAT[a]

Characteristic	% of expected nonresponders			% of expected responders, group IV (n = 246)[b,c]
	Group I (n = 114)[c]	Group II (n = 21)[c]	Group III (n = 66)[c]	
MAT = SAT[d]	84.1	95.2	43.9	46.7 (1.2)
MAT > SAT[e]	15.7	4.7	51.5	23.6 (2.8)
MAT < SAT[f]	0	0	4.5	21.9 (3.6)

[a] Animals were divided into groups according to the following categories: I, unvaccinated ewes and goats; II, unvaccinated ewe lambs and goat kids; III, vaccinated ewes and goats; and IV, vaccinated ewe lambs. Percentages were calculated according to the closest rounded decimal value.

[b] Numbers in parentheses represent percentages of animals which were vaccinated between 20 and 40 days prior to the experiment but did not react at positive titers in either test.

[c] n, number of serum samples tested per experiment.

[d] MAT result was equal to SAT result.

[e] MAT result was greater than SAT result.

[f] MAT result was less than SAT result.

included unvaccinated ewe lambs and goat kids. For group I, which included unvaccinated ewes and goats, agreement between the two methods was 84.1%.

For group III, which included vaccinated ewes and goats after their first gestation, a significantly lower level of agreement of 43.9% was observed ($P < 0.001$).

For groups I and II, titers in the MAT higher than those in the SAT, though in the negative range, were demonstrated for 15.7 and 4.7% of the serum samples, respectively. Titers in the SAT higher than those in the MAT were not observed. For group III, 51.5% of the tests demonstrated titers in the MAT higher than those in the SAT and 4.5% showed the opposite result.

For expectedly positive reactors (group IV) the agreement between MAT and SAT titers was 46.7%. The distributions for this group of MAT titers greater than SAT titers and SAT titers greater than MAT titers were almost equal (23.6 and 21.9%, respectively). Numbers in parentheses represent percentages of animals which were vaccinated prior to the experiment but nevertheless did not react at positive titers in either test.

The MAT and SAT results, obtained with the nonresponder and responder groups, were analyzed by a paired *t* test. Arbitrary values with inconsistent incremental steps were given to each serum titer (see Materials and Methods). Then the accumulated MAT values were subtracted from SAT values, and the difference between the two methods was calculated. The mean difference for the nonresponding group was $\bar{x} = -2.86$ (standard deviation = 6.1), showing a significant tendency of MAT readings to be higher than those of the SAT ($P < 0.001$). For the group of responding animals the mean difference was $\bar{x} = -0.63$ (standard deviation = 23.3; $P = 0.66$).

The correlation between the two methods was evaluated by using two criteria. Firstly, all data were included irrespective of being among positive or negative titers. Secondly, the results were compared at the individual sample level by testing differences in the titers obtained by each method. By using the Spearman correlation to test the data according to the first criterion, a very high and significant correlation factor ($r = 0.95$; standard estimation = 0.014) was found ($P < 0.001$). This correlation was based on 97.4 and 97.8% parities between the SAT and the MAT found for the negative titers and the positive titers, respectively.

Figure 1 depicts results obtained by interpretation of the data according to the second criterion. As can be seen in the figure, a very high and significant correlation factor of ($r = 0.96$; standard estimation = 0.005) was found ($P < 0.001$). At antibody titers below the cutoff point MAT results were higher than SAT results for 25 and 6 samples from groups II and III, respectively. For these groups SAT titers which exceeded MAT titers were observed with three and four samples, respectively.

At antibody titers interpreted as positive (groups IV and V), more samples with MAT titers higher than SAT titers were observed. MAT titers were higher in 5 and 17 samples for these groups, and they were lower in only 7 samples (group IV). Analysis of all the results by the general linear model similarly demonstrated a significantly ($P = 0.03$) higher mean for MAT titers ($\bar{x} = 67.4$; standard estimation = 0.58) than for SAT titers ($\bar{x} = 65.7$; standard estimation = 0.58).

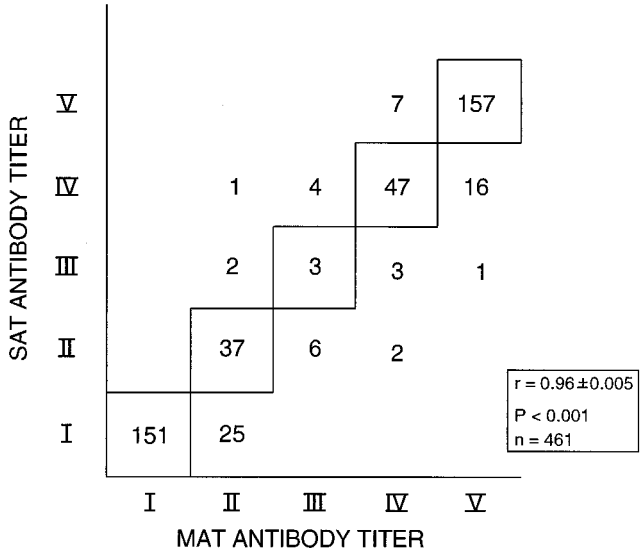

FIG. 1. Correlation between the MAT and the SAT according to the measured antibody titers. The numbers indicate all samples tested with the following results: I, negative results; II, antibody titers of 1:10 to 1:20 (+3); III, antibody titers of 1:20 (+4) to 1:40 (+1); IV, antibody titers of 1:40 (+2) to 1:40 (+4); and V, antibody titers of ≥1:80. n, number of tests performed.

DISCUSSION

In most countries where a brucellosis control program has been implemented, the CFT is the leading technique of diagnosis, with the SAT in second place. The specificity and sensitivity of the CFT were established as being superior to those of the SAT, being in the ranges of 95 and 80%, respectively (12, 19, 20). However, the SAT was superior to the CFT in the diagnosis of the earlier phases of the disease because of its capacity to detect immunoglobulin antibodies of the immunoglobulin M isotype which predominate in the beginning of the infection (6, 18, 25).

The use of the SAT as a diagnostic tool in spite of its recognized limitations has led to controversy concerning its importance. Several laboratories still prefer to use this test because of its simplicity compared with the CFT technique. However, the drawback of lowered sensitivity and specificity as well as the requirement of larger amounts of antigen for this method, and the nonsuitability of automation to facilitate diagnosis, makes the test unattractive. Instead, a MAT with qualities of improved sensitivity and specificity has been developed (3, 22, 30).

Recently, the Israeli Veterinary Services initiated an interim program for control of brucellosis in the small ruminant population. The program combines massive vaccination of the female lambs and goat kids with conventional doses of the live-vaccine *B. melitensis* strain Rev1 and eradication of infected animals. A major component in the success of such a program is the implementation of more than one diagnostic method to identify as many infected animals as possible.

Our aim in the present study was to comprehensively compare the MAT and the SAT, in order to evaluate their applicability as alternative methods in surveillance programs. The results showed higher levels of specificity for MAT and SAT (98.5 and 100%, respectively) (Table 1) than are usually reported (6, 12, 29).

A low-level specificity result for the agglutination test has usually been explained on the basis of two arguments. Firstly, the binding of nonspecific agglutinins to brucella cells has been suggested as contributing to positive responses in the SAT. This problem could be overcome by the addition of EDTA to the agglutination suspension (13, 21, 26). Secondly, agglutinating antibodies could be elicited in animals infected with other pathogens which present antigenic cross-reactivity with bru-

cella, such as *Yersinia enterocolitica* O:9 and *Escherichia coli* O:157 (8, 20, 23). However, such nonspecific reactions were not observed if animals from flocks free of the other diseases were chosen. Under these conditions, the reported specificity of the SAT was very high, with values up to 100% (10, 17, 29, 31, 32). Therefore, the high specificity shown in our results could also have been rationalized by similar arguments.

As shown by the data presented in this paper, one ewe had a positive reaction in the MAT but not in the SAT (Table 1, group II). While this result was not repeated with the other samples, it led to the calculation of a reduced specificity of the MAT compared with the SAT. However, the ewe's serum was also positive by two confirmatory tests, the CFT and the RBT. This may suggest that the MAT has actually identified a positive reactor among negative ones, whereas the SAT produced a false-negative result for this sample. Therefore, it can be concluded that the MAT's specificity was underestimated in our calculations because of the inclusion of this result as a false-positive one. Moreover, the sensitivity of the SAT was also miscalculated because a false-negative result was counted as a true-negative one. This conclusion, however, stands in contrast to the finding that both methods showed equal levels of sensitivity (93.9%) (Table 1).

Several statistical tests were carried out to better characterize the differences between the SAT and the MAT. An applied χ^2 test has shown MAT titers significantly higher than SAT titers for the group of nonresponding ewes and goats with a vaccination history (Table 2, group III; $P < 0.001$). Similarly, a paired *t* test and general linear model analyses have suggested that the sensitivity of the MAT was slightly higher than that of the SAT for the borderline titers distinguishing between positive and negative reactors (Fig. 1).

Disagreement between the methods was also found for serum samples obtained from positive reactors (Table 2, group IV). However, the proportions of serum samples in this group with MAT titers greater than SAT titers and those with SAT titers greater than MAT titers were similar (23.6 and 21.9%, respectively). One may conclude, therefore, that in the range of positive titers neither method gives a conclusive predictive value for the serological result.

The successful implementation of a brucellosis control program is dependent on the identification of all positive responders. For problematic flocks, the higher sensitivity shown by the MAT at the lower antibody titer levels, while not different from the sensitivity of the SAT in the positive range, makes the technique more favorable than the SAT. Also, the MAT can easily be adapted for performance by automatic equipment requiring the use of only small amounts of antigen per test. Therefore, this technique was chosen in Israel as the supplementary method to the CFT for routine diagnosis of ovine, caprine, and bovine brucellosis. Problematic cases of human brucellosis are also being tested by this method.

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