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Comparative Evaluation of the Enzyme-Linked Immunosorbent Assay in the Laboratory Diagnosis of Brucellosis

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An enzyme-linked immunosorbent assay was adapted to measure total and *Brucella abortus*-specific immunoglobulin M antibodies. The results were compared with those of conventional serological tests for *B. abortus* antibody on the sera of a number of normal controls, apparently healthy occupationally exposed workers, and patients with suspected acute brucellosis. Relative to other tests, the *B. abortus* enzyme-linked immunosorbent assay was found to be both highly sensitive and highly specific. The serological results obtained in occupationally exposed workers indicate a higher "normal range" for this group and therefore a possibility of false-positive results and overdiagnosis. It is therefore important to establish a separate "normal range" for occupationally exposed workers. Investigation of patients with acute brucellosis showed that the enzyme-linked immunosorbent assay for immunoglobulin M was the most sensitive serodiagnostic test and was likely to be of value in the serodiagnosis of acute brucellosis in occupationally exposed workers.

There is some uncertainty about the clinical aspects of human brucellosis. Although many of the infections, especially subacute and chronic, may be atypical, a pyrexia of unknown origin may be a possible diagnosis of brucellosis.

Brucella abortus is only isolated from a minority of infected patients (8), most of whom have an acute infection. Furthermore, positive cultures are usually obtained after 7 to 12 days of incubation; therefore, a positive diagnosis is more frequently made serologically.

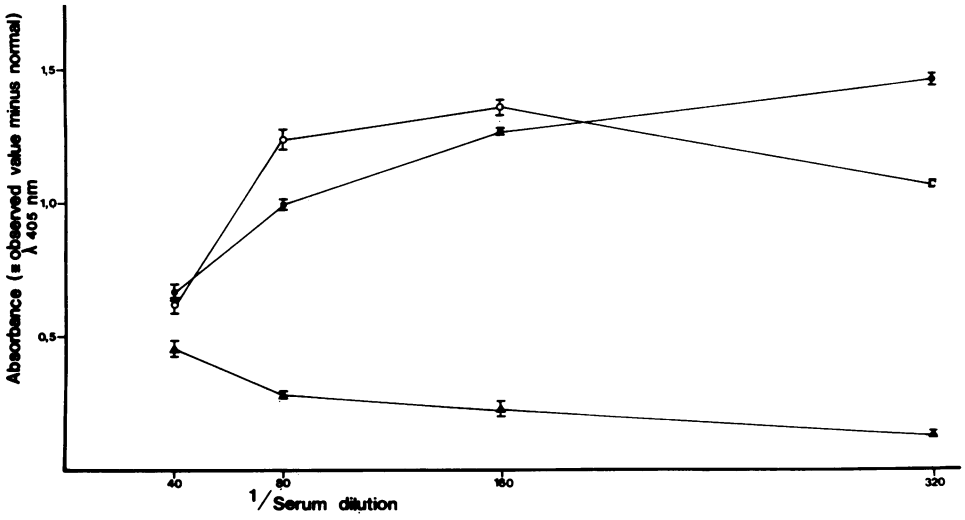

FIG. 1. Effects of serum dilution on absorbance with polyvalent (IgG plus IgM plus IgA) alkaline phosphatase conjugate. Symbols; ●, high positive; ○, mid positive; ▲, normal control.

There is much conflicting opinion about the nature of the humoral immune response and specific antibody class involved at a particular stage of the disease. It was first shown (6) that 2-mercaptoethanol-sensitive agglutinating antibodies (immunoglobulin M [IgM]) may be present in the sera of healthy individuals, whereas individuals with chronic brucellosis only had 2-mercaptoethanol-resistant antibodies (IgG).

Earlier studies have shown that the direct mercaptoethanol agglutination tests, complement fixation test (CFT), and anti-human immunoglobulin test are not always easy to interpret, since none is specific for a single immunoglobulin class and several tests are required for what is often merely a screening procedure. With the development of more sophisticated tests such as the indirect immunofluorescence test (IIF) (2), radioimmunoassay (5), and enzyme-linked immunosorbent assay (ELISA) (4), individual classes of *B. abortus*-specific antibodies could be detected. The frequency with which each class of antibody was detected in these studies seemed to depend on the type of subject studied and the sensitivity of the technique used. However, the results suggested that the presence of *B. abortus*-specific

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TABLE 1. Mean of the correlation coefficients obtained in three experiments

Absorbance value/antigen concentration	Mid-positive r^a	High-positive r
1:10	0.84	0.95
1:20	0.85	0.95
1:40	0.85	0.91
1:80	0.93	0.92
1:160	0.79	0.93
1:320	0.62	0.91

[a] r, Correlation coefficient.

TABLE 2. Variation in the *Brucella* ELISA

Alkaline phosphatase conjugate	Expression of results[a]	F well-to well	F day-to-day
Polyvalent	A	11.01	10.17
	B	5.02	9.00
	C	4.01	1.30
IgM	A	1.79	68.72
	B	2.39	85.44
	C	2.56	0.67

[a] A, Positive control without subtraction of negative value; B, positive control with subtraction of negative value; C, results expressed in A_{CR} units.

IgM is associated with recent exposure to the organism and acute infection and that IgM is replaced by IgG after repeated exposure or chronic infection.

The present investigation was undertaken to develop an ELISA for the serodiagnosis of brucellosis and to assess its reliability relative to existing serological tests.

(This is an extract from a dissertation by E. De Klerk for the degree of Master of Science in Medicine, University of the Witwatersrand, Johannesburg, South Africa.)

MATERIALS AND METHODS

Serum specimens. (i) Control sera. A total of 150 serum samples, consisting of 50 samples from healthy laboratory personnel or medical students and 100 from black Africans from South West Africa who were being used as part of an epidemiological survey served as the control group. The *B. abortus* ELISA and IIF were used to screen for the presence of total *B. abortus* antibodies in all the controls. *B. abortus*-specific IgM (ELISA and IIF) was also measured in the first 50 controls.

(ii) Sera from occupational workers. Sera taken from 100 apparently healthy occupationally exposed workers (occupational workers) (Research Institute for Veterinary Science, Onderstepoort, South Africa) and received for routine investigation were used in this study. In addition to the conventional tests (CFT, standard agglutination test [SAT], and IIF), all sera were tested by ELISA for total specific antibodies and *Brucella*-specific IgM. Workers whose sera gave positive results with two or more of the conventional tests were referred for medical examination. Immunoglobulins in serum and full blood counts were also investigated in this group.

(iii) Sera from patients with active brucellosis. Sera from four patients were provided by A. C. Mauff (South African Institute for Medical Research, Johannesburg, South Africa). All but one were abattoir workers and were clinically diagnosed as having acute brucellosis. The exception was a patient with no obvious history of previous contact with *B. abortus*, who was considered a doubtful case.

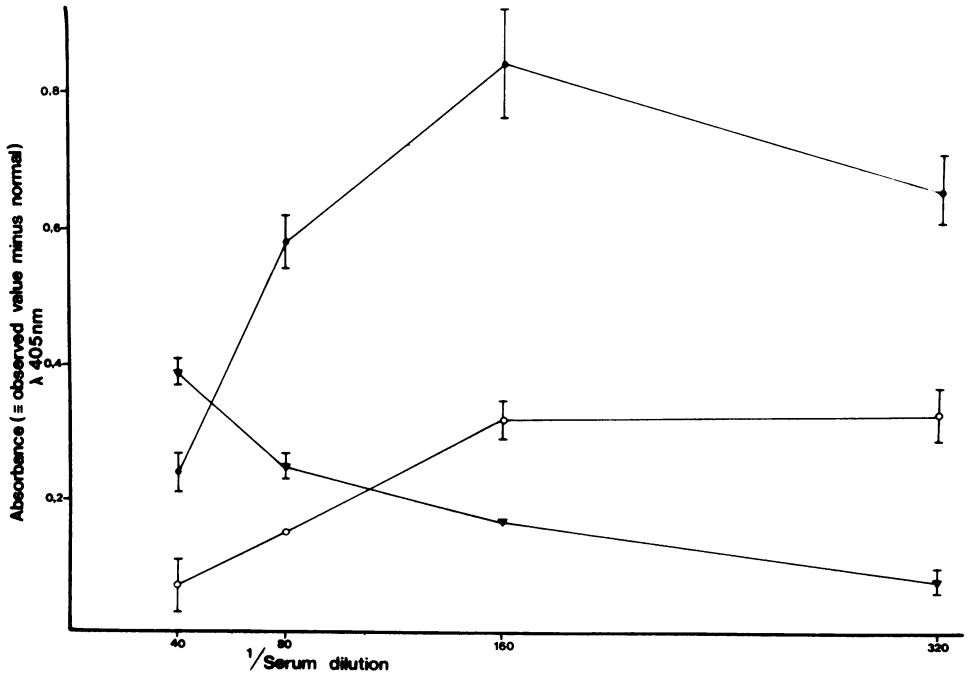

FIG. 2. Effects of serum dilution on absorbance with IgM alkaline phosphatase conjugate. Symbols: ●, high positive; ○, mid positive; ▼, normal control.

Methods for the measurement of *B. abortus* antibodies. (i) Conventional serological tests. The SAT, CFT, and IIF were performed by routine serological procedures.

(ii) *B. abortus* ELISA test. The ELISA was performed essentially as described previously (1). In this procedure the *B. abortus* SAT antigen (heat-killed S99 laboratory strain; Research Institute for Veterinary Science, Onderstepoort) was diluted 1:80, and all sera were screened in duplicate at a 1:320 dilution as determined by chequerboard titration (Fig. 1, Table 1). Uncoated wells served as background controls.

Absorbance values were registered spectrophotometrically at a wavelength of 405 nm (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). After subtraction of the background counts, a mean of the two readings was obtained and the results were expressed as correct absorbance (A_{CR}) values: $A_{CR} = (A_t - A_n) \times \{[\text{mean } (A_p - A_n)]/[(A_p - A_n) \text{ on test day}]\} \times 100$, where A_t, A_p, and A_n are the absorbance values of the unknown, positive, and negative control sera. The control sera were selected on the basis of the SAT, CFT, and IIF results. The mean $(A_p - A_n)$ was taken as the mean difference between the positive and negative control sera over a period of several months.

Methods of tests used to investigate possible sources of false-positive reactions occurring in the *B. abortus* ELISA technique. The *Yersinia enterocolitica* SAT and measurements of C-reactive protein (CRP) and rheumatoid factor (RF) were performed by routine serological procedures.

***Y. enterocolitica* ELISA technique.** The basic procedure was the same as for the *B. abortus* ELISA technique. In addition to a 1:80 antigen dilution, microtiter plates were also coated with a 1:20 and 1:40 dilution of *Yersinia* O-V (previously designated serotype 9) antigen (SAIMR, Johannesburg, South Africa) to eliminate the possibility of higher cross-reactivity at lower antigen concentrations. For the same reason, a serum dilution of 1:320 was chosen to evaluate possible cross-reactions with *Yersinia* O-V antigen when sera were screened for *B. abortus* antibodies at this dilution. The absorbance values are expressed as $A_t - A_n$.

Absorption of RF. RF-positive sera were allowed to react with latex-RF-reagent (at a final serum dilution of 1:40; Behringwerke, Marburg, West Germany) for 20 to 25 min at 22°C. After centrifugation at 2,000 rpm for 15 min, the supernatant was diluted 1:8 in phosphate-buffered saline–Tween 20 containing 0.4% bovine serum albumin. The absorbed sera were then available for testing by the standard ELISA procedure.

TABLE 4. Differences in variability in IgM ELISA results

Control	Mean ± SD A_{450}	CV (%)[a]
Normal	0.090 ± 0.01	11.1
Positive	0.750 ± 0.02	2.7
False-positive	0.185 ± 0.088	47.3

[a] Coefficient of variation (standard deviation of observations divided by the mean value, expressed as a percentage).

RESULTS

Effects of antigen concentration and serum dilution on absorbance. The optimal antigen concentration and serum dilution for the *B. abortus* ELISA were determined by a checkerboard titration of a normal, mid-positive, and high-positive control serum (classified on the basis of the SAT, CFT, and IIF results; data not shown). Expression of results as the mean and standard errors obtained in three experiments over a period of 3 days made it difficult to assess the point of maximum antibody binding (data not shown). For this reason, the correlation coefficients were calculated (on linear regression models) for each antigen concentration (Table 1). An antigen concentration of 1:80 was adopted, since it gave a high correlation (0.93) for the mid-positive control serum.

A serum dilution of 1:320 was found to be optimal for both polyvalent and IgM alkaline phosphatase conjugate procedures, since it gave the best separation between the high-positive, mid-positive, and normal control sera (Fig. 1 and 2).

Assessment of well-to-well and day-to-day variability. In three experiments over a period of 3 days, a positive and a negative control serum were tested over 16 antigen-coated wells. Table 2 shows the results obtained when these data were subjected to the F test. The critical value for F was as follows: well-to-well variation, F (0.05) (7.14) = 2.76; day-to-day variation, F (0.05) (2.14) = 3.74. Expression of results for polyvalent alkaline phosphatase conjugate procedures as A_{CR} units eliminated the day-to-day but not the well-to-well

TABLE 3. ELISA results in RF-positive sera

Specimen no.	RF titer	ELISA value (A_{CR} units) for:	
		IgG + IgM + IgA	IgM
1	1:320	0	0
2	1:40	0	0
3	1:320	0	0
4	1:160	0	0
5	1:160	0	0
6	1:640	0	0
7	1:160	0	0
8	1:320	14	35[a]
9	1:1,280	12	0
10	1:80	3	0
11	1:640	0	6
12	1:2,560	0	0
13	1:5,120	15	59[a]
14	≥1:10,240	0	0
15	≥1:10,240	4	2

[a] A_{CR} units > 25.

TABLE 5. Elimination of false-positive *Brucella* ELISA results by latex-RF absorption

Serum sample	IgM ELISA (A_{CR} units) for:	
	Unabsorbed sera	Absorbed sera
Normal controls		
1	7	5
2	4	5
3	4	5
4	9	9
Positive controls		
1	68	52
2	47	35
3	38	30
RF-positive sera		
1	29	6
2	40	14

TABLE 6. Serological cross-reactions between *B. abortus* and *Y. enterocolitica* O-group V

Case	*Brucella* SAT value	*Yersinia* SAT value	*Brucella* ELISA value	*Yersinia* ELISA value[a] at:		
				1:20	1:40	1:80
Cross-reacting controls						
1	1:50	1:50	0.296	0.000	0.000	0.000
2	1:100	1:20	0.570	0.000	0.000	0.000
3	1:40	1:10	0.462	0.000	0.000	0.000
4	1:160	1:160	0.807	0.450 ± 0.02	0.480 ± 0.001	0.440 ± 0.04
Positive brucellosis controls						
1	1:100	<1:10	0.436	0.000	0.000	0.000
2	1:400	<1:10	1.250	0.000	0.000	0.000
3	1:400	<1:10	1.190	0.000	0.000	0.000
4	1:800	<1:10	1.200	0.000	0.000	0.000
Doubtful brucellosis cases						
1	<1:10	<1:10	0.468	0.000	0.000	0.000
2	<1:10	<1:10	0.265	0.000	0.000	0.000
3	<1:10	<1:10	0.469	0.000	0.000	0.000
Positive yersiniosis controls						
1	1:10	1:100	0.063	0.690 ± 0.11	0.700 ± 0.08	0.760 ± 0.01
2	1:40	1:100	0.056	0.320 ± 0.02	0.320 ± 0.03	0.300 ± 0.02
3	1:40	1:50	0.030	0.333 ± 0.07	0.330 ± 0.07	0.400 ± 0.03
4	<1:10	1:800	0.004	0.960 ± 0.1	0.980 ± 0.03	1.060 ± 0.02

[a] Mean ± standard error of two experiments.

variability, although the latter was improved. When IgM results were expressed as A_{CR} units, no significant well-to-well or day-to-day variability was apparent.

Possible sources of false-positive *B. abortus* ELISA results. (i) CRP as a source of false-positive results. Since all 15 sera tested (CRP levels, 10 to ≥189 μg/ml) gave negative results (results not shown), it was concluded that CRP has no effect on the *Brucella* ELISA test.

(ii) RF as a source of false-positive results. The capacity of RF to cause false-positive results for IgM has been previously recognized in ELISA techniques (7). All sera gave values of less than 25 A_{CR} units (negative ELISA value, as determined by data shown in Table 8) when polyvalent alkaline phosphatase conjugate was used, whereas specimens 8 and 13 gave elevated IgM values (Table 3). The SAT, CFT, and IIF were negative, confirming that these were true false-positive results.

False-positive IgM ELISA results due to the presence of RF were also found to be more variable (Table 4). The coefficient of variation was elevated to 47.3% in the RF-positive serum, whereas a true-positive was elevated to only 2.7%. This phenomenon is possibly associated with the specificity of the reaction.

To eliminate the false-positive IgM ELISA results, the technique described above was used. The results are shown in Table 5. The absorption step had no effect on normal control sera, whereas RF-positive sera became negative. A possible explanation for the decrease in the A_{CR} units observed in positive controls could be that the latex-RF-reagent also removes, albeit to a lesser degree, specific IgM.

Serological cross-reactions between *B. abortus* and *Y. enterocolitica* O-group V (serotype 9). The results of the study of serological cross-reactions between *B. abortus* and *Y. enterocolitica* are presented in Table 6. Doubtful brucellosis patients included those whose sera gave negative results for the *Brucella* SAT and IIF as well as for the *Yersinia* SAT, but gave positive results for the *Brucella* CFT and ELISA.

None of the positive or doubtful *Brucella* controls gave false-positive *Yersinia* ELISA values, nor did any *Yersinia* controls give positive values for the *Brucella* ELISA. In one of the four cross-reacting controls, however, the ELISA value for *Y. enterocolitica* was approximately half that obtained for *B. abortus*. Retesting of this serum, after absorption with 1 volume of packed *Y. enterocolitica* O-group V cells and 3 volumes of serum (37°C for 18 h), produced a similar ELISA value (0.755) and a *Brucella* SAT of 1:80, whereas the *Yersinia* SAT became negative. A possible explanation for this observation could be the presence of both *Yersinia* and *Brucella* antibodies.

Evaluation of the *B. abortus* ELISA test in comparison with the SAT, CFT, and IIF tests. Four different techniques (SAT, CFT, IIF, and ELISA) were used for the serodetection of antibodies against *B. abortus* in serum samples from 100 occupational workers. The results of these tests are shown in Table 7, which also gives an account of the number of positive specimens obtained when different ELISA A_{CR} units are taken as the upper limits of normal antibody

TABLE 7. Seropositive results obtained in 100 occupational workers received for routine investigations of *B. abortus* antibodies

Test	No. of positive specimens	Mean ± SE of positive results
SAT (≥1:50)[a]	12	245.33 ± 80.91
CFT (≥1:5)	12	30.24 ± 7.92
IIF (≥1:10)		
IgG + IgM + IgA	13	102.31 ± 34.94
IgM	5	26.00 ± 6.00
ELISA[b]		
A_{CR} 20		
IgG + IgM + IgA	20	
IgM	7	
A_{CR} 25		
IgG + IgM + IgA	16	71.44 ± 9.74
IgM	5	46.20 ± 8.19
A_{CR} 30		
IgG + IgM + IgA	14	
IgM	3	

[a] Positive titer.
[b] No. of positive sera at different A_{CR} units.

concentration. Data shown in this table were analyzed to assess the sensitivity, specificity, positive predictive value, and negative predictive value of each laboratory assay (as calculated by standard formulae [3]).

In addition to the standardization criteria previously described (1), one specimen which gave positive results with the IFF (IgG plus IgM plus IgA) was considered a true-positive since IIF was the only reliable conventional test used that is capable of detecting non-agglutinating antibodies. The IIF results for *B. abortus*-specific IgM were taken as standard, since this was the only conventional test specific for IgM.

From the results shown in Table 8, an ELISA value of 25 A_{CR} units was chosen as the upper limit of normal antibody concentration for both total *Brucella* antibody and specific IgM determinations. The IIF test results are not shown, since they were equivalent to the standardized results.

Other investigations. All CRP values, full blood counts, serum immunoglobulins, and clinical investigations were normal (results not shown). Of the 100 sera investigated, 3 gave positive RF titers (1:40 to 1:160), although they were all negative for *B. abortus*-specific IgM with both the ELISA and the IIF. Possible explanations for these findings could be the increased variability of sera containing RF or the low levels of RF in these sera.

The results of the study to investigate the effect of antibodies against *Y. enterocolitica* O-group V on the *B. abortus* ELISA are as shown in Table 6.

Results in normal controls. All of the 150 sera tested gave negative results with the IIF and ELISA for total *B. abortus* antibody as well as the IIF for *Brucella*-specific IgM. Only one false-positive IgM ELISA result occurred (40 A_{CR} units) in a specimen containing RF (1:320). Absorption with latex-RF-reagent reduced this value to 14 A_{CR} units. The ELISA results of the two control groups are shown in Table 9.

Serological results in patients with clinically active brucellosis. The serological results in patients with clinically active brucellosis are shown in Table 10. Patient 1 was the doubtful brucellosis case; the others presented with an acute pyrexia and were diagnosed as having acute brucellosis. Only patient 3 had a positive blood culture, since treatment of the others was started before the bacteriological investigations. Patients 2 and 4 responded well to therapy, but patient 3 died from a subacute bacterial endocarditis, and *B. abortus* was isolated from the heart valves (A. C. Mauff, personal communication).

TABLE 8. Relative sensitivities, specificities, positive predictive values, and negative predictive values of different techniques used for measurement of *B. abortus* antibodies

Test	% Sensitivity	% PPV[a]	% Specificity	% NPV[a]
SAT	92.3	100	100	98.9
CFT	76.9	48	87.4	96.2
IgG + IgM + IgA ELISA				
A_{CR} 20	100	65	92	100
A_{CR} 25	100	81.3	97	100
A_{CR} 30	92.3	85.7	98	98.8
IgM ELISA[b]				
A_{CR} 20	100	71.4	98	100
A_{CR} 25	100	100	100	100
A_{CR} 30	60	100	100	97.9

[a] PPV, Positive predictive value; NPV, negative predictive value.
[b] As calculated when IgM IIF results are taken as standard.

TABLE 9. *Brucella* ELISA results in 150 normal controls

Control group	No. of serum samples	ELISA (IgG + IgM + IgA)		ELISA (IgM)	
		A_{CR} units ≤ 1	A_{CR} units >1≤21	A_{CR} units ≤1	A_{CR} units >1≤5[a]
Laboratory personnel or medical students	50	40 (80)[b]	10 (20)	48 (96)	1 (2)
Sera from South West Africa	100	79 (79)	21 (21)	ND[c]	ND

[a] False-positive specimen excluded.
[b] Percentage.
[c] ND, Not done.

DISCUSSION

This study has shown the ELISA to be a sensitive and specific test for the detection of *B. abortus* antibodies.

The *B. abortus* SAT was of high specificity and reasonable sensitivity and is therefore a useful confirmatory test. The CFT, however, had a sensitivity of 76.9%, of which only 48% were true-positive results (Table 8). The combined ELISA (polyvalent) detected more positive sera than did either the SAT or CFT alone and was comparable with the IIF.

A noteworthy point is the susceptibility of ELISA measurements of IgM in serum to the influence of high titers of RF in serum, which may cause false-positive results. Although this association is well known, the *B. abortus* ELISA for total antibodies was not affected by RF. Laboratories receiving large numbers of specimens for numbers of specimens for *B. abortus* ELISA determinations would be well advised to screen sera for total *B. abortus* antibodies and subsequently test positive sera for specific IgM.

Examination of sera from apparently healthy occupational workers showed that almost any combination of positive results may occur in people exposed to *B. abortus* (Table 7). These results indicate a higher "normal range" for this group and hence a possibility of false-positive results and overdiagnosis when compared with an unexposed control group. A separate normal range should therefore be established for occupational workers before a correct diagnosis of brucellosis can be confirmed. Investigation of clinically active brucellosis patients showed the IgM ELISA to be more sensitive than the IIF (Table 10). Not only did the ELISA detect *B. abortus*-specific IgM in all three active cases, but the A_{CR} units obtained were higher than those found in the occupational group and the IIF titers were similar.

Although only a few acute brucellosis cases were available for investigation, our results show that the *B. abortus* IgM ELISA may be of value in the serodiagnosis of acute

TABLE 10. Serological results obtained in four patients clinically diagnosed as possible brucellosis cases

Patient no.	SAT	CFT	IIF		ELISA A_{CR} units	
			IgG + IgM + IgA	IgM	IgG + IgM + IgA	IgM
1	1:320	ND[a]	1:40	<1:10	50	0
2	1:1,280	≥1:512	1:160	1:10	58	131
3	1:20,480	≥1:512	1:80	1:20	77	135
4	1:320	ND	1:40	<1:10	38	32

[a] ND, Not done. Insufficient serum available before treatment.

brucellosis as a means of detecting early-stage infections and differentiating between active and subclinical (asymptomatic) cases in occupationally exposed people.

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