

Evaluation of Seven Tests for Diagnosis of Human Brucellosis in an Area Where the Disease Is Endemic[∇]

M. Concepción Gómez,^{1*} José A. Nieto,² Carmen Rosa,² Paloma Geijo,²
M. Ángeles Escribano,³ Amparo Muñoz,³ and Clara López³

Microbiology Laboratory Hospital Severo Ochoa (Leganés), Madrid,¹ and Department of Internal Medicine² and Microbiology Laboratory,³ Hospital Virgen de la Luz, Cuenca, Spain

Received 27 October 2007/Returned for modification 4 December 2007/Accepted 17 April 2008

The results of seven serologic tests for diagnosis of human brucellosis were evaluated. The titrated Rose Bengal test, microagglutination test, microtiter-adapted Coombs test, and immunocapture-agglutination test (Brucellacapt) were positive for all sera from patients with acute brucellosis. The immunoglobulin G (IgG), IgM, and IgA commercial enzyme immunoassays (ELISAs) failed to show specific antibodies in 3 patients, 10 patients, and 1 patient, respectively. The sensitivity of ELISA is not higher than that of conventional tests.

Brucellosis is an endemic zoonotic disease in many parts of the world, notably in Mediterranean countries and the Middle East. The diagnosis of brucellosis is made by the isolation of *Brucella* species (i.e., in blood cultures), but this method is successful in only 40 to 70% of cases (18). Therefore, laboratory diagnosis of brucellosis very often relies on detecting specific serum antibodies (5, 19). Several serological tests have been used for the diagnosis of human brucellosis. The serum agglutination test (SAT) for brucellosis, developed by Wright et al. in 1897 (17), is still the reference to which other tests are compared. Other notable tests that have been developed since then are the Rose Bengal test, complement fixation test, indirect Coombs test, enzyme immunoassay (ELISA) (6, 15), and, more recently, an immunocapture-agglutination test (Brucellacapt) (10). However, the interpretation of these tests is often difficult in areas of endemicity in which a large part of the population has contact with animals or products of animal origin and could develop antibodies against *Brucella*. In this study, the results obtained with seven different tests for detection of *Brucella*-specific antibodies in an area of endemicity were analyzed. A 12-month clinical and serologic follow-up was performed after the treatment was started. As a reference, the antibody levels in the healthy population of that area were also tested.

One hundred twenty serum samples from 25 patients with acute brucellosis and 90 from healthy individuals (blood donors) were included in this study. The diagnosis of brucellosis was based on clinical findings and on either positive blood cultures for *Brucella* or the presence of serum antibodies (SAT titer \geq 160). At least three blood cultures were drawn from each patient at diagnosis. Follow-up cultures were drawn at the end of the treatment and 3, 6, and 12 months later. For four patients the 12-month cultures were not performed. *Brucella* was identified according to Munich's taxonomy criteria (8). Serum samples were collected on admission and 1, 3, 6, and 12

months later. For four patients the 12-month control sample was not assayed. For the group of blood donors only one serum sample was analyzed. The titrated Rose Bengal test, microagglutination test (MAT), microtiter-adapted Coombs test, Brucellacapt, and ELISAs for immunoglobulin M (IgM), IgG, and IgA antibodies were performed on each serum sample. The microtiter-adapted Coombs test was not performed for the group of healthy individuals. The Rose Bengal test was performed with commercial *Brucella abortus* antigen (Bio Systems, Barcelona, Spain), according to the manufacturer's instructions. Titrations were made by serial twofold dilutions with saline solution. The MAT was performed in rigid U-bottomed microtiter plates as described by Bettelheim et al. (3). The antigen used was a commercial suspension of *Brucella abortus* (Bio-Rad, Marnes-la-Coquette, France). A microtiter-adapted Coombs technique was performed as described by Otero et al. (12). The Brucellacapt test (Vircell SL, Granada, Spain) was performed as specified by the manufacturer. IgG, IgM, and IgA ELISAs were performed using a commercial kit (Serion/Virion, Würzburg, Germany). The testing procedure followed exactly the manual of instructions. In order to evaluate ELISA antibody concentration, a highly specific standard curve as well as a highly specific evaluation table included in each test kit was used. Results are shown in arbitrary units per milliliter of serum by extrapolating the absorbance values by means of a standard curve established with an internal reference sample. Titers over 30 U for IgG, over 20 U for IgM, and over 15 U for IgA were considered positive by the manufacturer. Sensitivity, specificity, and positive and negative predictive values were calculated by using a standard formula (7). SPSS software (version 13.5; SPSS Inc., Chicago, IL) was used for the statistical management of the data.

Twenty-five patients were included in the study; 22 were male and 3 were female, and their ages ranged from 12 to 80 years (median, 41 years). Twenty-two patients lived in a rural habitat, and 24 patients reported exposure to animals or their products. In 24 cases (96%) it was possible to identify at least one risk factor for brucellosis: close contact with animals (96%), ingestion of raw milk (4%), or work in an abattoir (64%). Fever was present in 76% of patients, and 44% pre-

* Corresponding author. Mailing address: Servicio de Microbiología, Hospital Severo Ochoa, Avda. de Orellana s/n, 28911 Leganés, Madrid, Spain. Phone: 34 91 4818441. Fax: 34 914818442. E-mail: mcongocri@hotmail.com.

[∇] Published ahead of print on 30 April 2008.

TABLE 1. Median serum antibody titers in 90 healthy individuals and 25 patients with brucellosis on admission and 1, 3, 6, and 12 months after therapy

Test	Blood donors (n = 90)	Titer ^a [range (median)] for:				
		Patients at:				
		Admission (n = 90)	1st mo (n = 25)	3rd mo (n = 25)	6th mo (n = 25)	12th mo (n = 21)
Titred RB	0-1:2 (0)	1:1-1:64 (1:16)	1:1-1:128 (1:4)	1:1-1:64 (1:2)	1:1-1:32 (1:1)	1:1-1:16 (1:1)
MAT	0-1:40 (0)	1:160-1:40,960 (1:2,560)	1:20-1:20,480 (1:640)	0-1:10,240 (1:320)	0-1:5,120 (1:160)	0-1:320 (1:80)
Coombs test	ND ^b	1:1,280-1:81,920 (1:10,240)	1:64-1:655,360 (1:5,120)	1:320-1:163,840 (1:5,120)	1:80-1:81,920 (1:2,560)	1:40-1:40,960 (1:2,560)
Brucellacapt	0-1:80 (0)	1:320-1:81,920 (1:20,480)	1:320-1:40,960 (1:2,560)	1:80-1:20,480 (1:640)	1:40-1:10,240 (1:640)	1:40-1:5,120 (1:640)
IgG ELISA	1-9 (1)	5-263 (73)	6-336 (104)	8-265 (88)	8-292 (58)	2-156 (65)
IgM ELISA	1-5 (1)	1-105 (47.5)	2-149 (16)	2-83 (22)	2-56 (8.5)	1-14 (6)
IgA ELISA	2-18 (2.5)	14-1,320 (164.5)	8-1,419 (111)	5-1,422 (69.5)	5-1,040 (53.5)	6-274 (62)

^a Dilution titers for the titrated Rose Bengal test (RB), MAT, Coombs test, and Brucellacapt; units for ELISAs.

^b ND, not done.

sented signs of focal infection. Blood cultures were positive in 13 cases (52%). The initial response to treatment was satisfactory in all patients. The titrated Rose Bengal test, the MAT, the Coombs test, and the Brucellacapt were positive for all sera from patients with acute brucellosis (titers \geq 1:1, 1:80, 1:1,280, and 1:320, respectively). The IgG, IgM, and IgA ELISAs failed to show specific antibodies in 3 patients, 10 patients, and 1 patient, respectively. For comparison purposes, the sera of 90 blood donors were also analyzed. Their ages ranged from 18 to 65 years (median, 32.5 years); 55 were female, and all of them lived in the same area. Sera from these healthy individuals were uniformly negative for IgG and IgM by ELISA. One serum showed a low IgA ELISA-positive result. The titrated Rose Bengal test, the MAT, and the Brucellacapt were positive for 3 sera (titer, 1:2), 1 serum (titer 1:40), and 12 sera (titers \leq 1:80) of blood donors, respectively. The median serum antibody titers from patients on admission and 1, 3, 6, and 12 months after treatment as well as from healthy individuals are shown in Table 1. The statistical analysis of the tests performed on admission are presented in Table 2. Specificity and positive predictive and negative predictive values are over 90% in all tests. IgM and IgG ELISAs have the lowest sensitivity (60% and 84%, respectively).

Overall, the results of our study are in accordance with classic concepts about the relevance of the MAT, Coombs test, and Brucellacapt test for diagnosis of human brucellosis. The serologic diagnosis of acute brucellosis is not definitively established. Many authors consider a SAT titer of \geq 1:160 to be indicative of active brucellosis (14, 20). However, active brucellosis cannot be excluded in patients with SAT titers lower

than 1:160. In our study two patients showed MAT titers of 1:80. These patients had Coombs test titers of \geq 1:10,240 and Brucellacapt titers of \geq 1:640. These data highlight the importance of using more than one test. Orduña et al. found a good correlation between the Brucellacapt and the Coombs test (10). Its simplicity makes Brucellacapt the most suitable complementary test for diagnosis of acute brucellosis.

Nevertheless, in regions where brucellosis is endemic, a large proportion of the population may have persistent *Brucella*-specific antibodies. Under such conditions, the interpretation of *Brucella* serologic tests may be difficult. In the study area, the MAT was positive for only one healthy blood donor, with a titer of 1:40. In contrast, all patients presented Brucellacapt titers of \geq 1:320. Thus, no healthy individuals had Brucellacapt titers of $>$ 1:80. Seroprevalence studies (1, 4) of *Brucella* antibodies performed in Saudi Arabia showed rates ranging from 4.4% (cutoff point, 1:320) to 11% (cutoff point, 1:160). Most recently, Kose et al. found a seroprevalence ranging from 0 to 5.6% in the Wright SAT (cutoff point, 1:100) in rural and suburban Anatolian communities, respectively (9). In Mexico, the seroprevalence in blood donors is 3.6% (16). These data show the importance of regional differences in the prevalence of antibodies to *Brucella* in countries in which the disease is endemic. It is therefore important to establish a "normal range" for the population of these countries.

Previous studies found ELISA to be an effective method for diagnosis of brucellosis (2, 11). In this study, using a commercial-reagent IgG ELISA, we failed to show any specific antibody in three patients. These patients presented MAT titers and Brucellacapt titers of \geq 1:2,560 and 1:5,120, respectively, as well as positive IgM and IgA ELISAs. Moreover, the IgM ELISA did not show specific antibodies in 10 patients. These patients had MAT titers and Brucellacapt titers over 1:80 and 1:640, respectively, and the IgG and IgA ELISAs were also positive. Because of its low sensitivity (60%), the commercial IgM ELISA has a limited value for diagnosis of acute brucellosis. Several antigens have been used in ELISAs of samples from patients with brucellosis. They are obtained from *Brucella abortus* or *Brucella melitensis* in the form of whole cells, sonic extracts of cells, lipopolysaccharides, or proteins. The variety of antigens used in the tests may lead to relevant differences among them, and lack of standardization could account for the discrepancies.

No patient relapsed, and serum antibody titers decreased

TABLE 2. Accuracy indices of the tests^a

Test	Sensitivity	Specificity	PPV ^b	NPV ^c
RB ^d	1.00	0.97	0.89	1.00
MAT	0.92 ^e	1.00	1.00	0.98
Brucellacapt	1.00	1.00	1.00	1.00
IgG ELISA	0.84	1.00	1.00	0.96
IgM ELISA	0.60	1.00	1.00	0.90
IgA ELISA	0.96	0.98	0.92	0.99

^a Cutoff points for positive tests were as follows: RB, \geq 1:1; MAT and Brucellacapt, \geq 1:160.

^b PPV, positive predictive value.

^c NPV, negative predictive value.

^d RB, Rose Bengal test.

^e Two patients had MAT titers of 1:80.

significantly during the follow-up. ELISA IgM titers decreased further than others. However, many patients showed persistently high Rose Bengal test, MAT, Coombs test, Brucellacapt, and ELISA IgG and IgA titers despite a satisfactory clinical outcome and negative blood cultures. The interpretation of this finding will be difficult to establish as long as the time until total intracellular eradication of *Brucella* cannot be precisely known and no accurate criteria for complete cure exist. In addition, persistently raised titers are a drawback for patients with signs or symptoms suggestive of brucellosis when they are caused by other infections or noninfectious diseases. Overdiagnosis and exposing patients to unnecessary anti-*Brucella* treatment may follow. This problem will continue until more-specific tests are developed, based on the detection of *Brucella* antigens or on the isolation of the organism (13).

In summary, the routine classic MAT and Brucellacapt test offer good results for the diagnosis of brucellosis in areas of endemicity when adequate cutoff points are used. Although an antibody titer of $\geq 1:160$ is common in patients with active infection, lower titers should not be disregarded. Conversely, MAT titers of $\geq 1:160$ and Brucellacapt titers of $\geq 1:320$ do not always indicate active brucellosis. ELISA is a rapid, sensitive, and specific assay, but its performance was not better than that of conventional tests. The commercial IgM ELISA showed low sensitivity.

REFERENCES

1. Al Skait, M. A. 1999. Seroepidemiological survey of brucellosis antibodies in Saudi Arabia. *Ann. Saudi Med.* **19**:219–222.
2. Araj, G. F., A. R. Lulu, M. Y. Mustafa, and M. I. Khateeb. 1986. Evaluation of ELISA in the diagnosis of acute and chronic brucellosis in human beings. *J. Hyg.* **97**:457–469.
3. Bettelheim, K. A., W. J. Maskill, and J. Pearce. 1983. Comparison of standard tube and microagglutination techniques for determining *Brucella* antibodies. *J. Hyg.* **90**:33–39.
4. Cooper, C. W. 1992. Prevalence of antibody to *Brucella* in asymptomatic well individuals in Saudi Arabia. *J. Trop. Med. Hyg.* **95**:140–142.
5. Díaz, R., and I. Moriyon. 1989. Laboratory techniques in the diagnosis of human brucellosis, p. 73–83. *In* E. J. Young and M. J. Corbel (ed.), *Brucellosis: clinical and laboratory aspects*. CRC Press, Boca Raton, FL.
6. Gad El-Rab, M. G., and A. M. Kambal. 1998. Evaluation of a *Brucella* enzyme immunoassay test (ELISA) in comparison with bacteriological culture and agglutination. *J. Infect.* **36**:197–201.
7. Ilstrup, D. M. 1990. Statistical methods in microbiology. *Clin. Microbiol. Rev.* **3**:219–226.
8. Jones, L. M., and W. Wundt. 1971. International Committee on Nomenclature of Bacteria. Subcommittee on the taxonomy of *Brucella*. *Int. J. Syst. Bacteriol.* **21**:126–128.
9. Kose, S., H. L. Smits, T. H. Abdoel, and Y. Ozbel. 2006. Prevalence of *Brucella* antibodies in rural and suburban communities in three provinces of Turkey: need for improved diagnosis and prevention. *J. Infect.* **53**:308–314.
10. Orduña, A., A. Almaraz, A. Prado, M. P. Gutiérrez, A. García-Pascual, A. Dueñas, M. Cuervo, R. Abad, B. Hernández, B. Lorenzo, M. A. Bratos, and A. R. Torres. 2000. Evaluation of an immunocapture-agglutination test (Brucellacapt) for serodiagnosis of human brucellosis. *J. Clin. Microbiol.* **38**:4000–4005.
11. Osoba, A. O., H. Balkhy, Z. Memish, M. Y. Khan, A. Al-Thagafi, B. Al Shareef, A. Al Mowallad, and G. A. Oni. 2001. Diagnostic value of *Brucella* ELISA IgG and IgM in bacteremic and non-bacteremic patients with brucellosis. *J. Chemother.* **13**(Suppl. 1):54–59.
12. Otero, J. R., A. Fuertes, E. Palenque, and A. R. Noriega. 1982. Microtiter-adapted method that facilitates the Coombs test for brucellosis. *J. Clin. Microbiol.* **16**:737–738.
13. Queipo-Ortuno, M. I., P. Morata, P. Ocon, P. Manchado, and J. D. Colmenero. 1997. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. *J. Clin. Microbiol.* **35**:2927–2930.
14. Rodríguez Torres, A., and J. Feroso. 1987. Brucellosis. *Medicine* **76**:3165–3177.
15. Rubio, M., B. Barrio, and R. Díaz. 2001. Usefulness of Rose Bengal, Coombs and counter-immunoelectrophoresis for the diagnosis of human brucellosis cases with negative seroagglutination. *Enferm. Infecc. Microbiol. Clin.* **19**:406–407.
16. Torres-Padilla, J. C., A. López-Merino, R. M. García-Escamilla, and J. N. Gutiérrez-García. 2004. Anti-*Brucella* antibody seroprevalence in blood donors for therapeutic ends at three blood banks of the Mexican Institute of Social Security. *Gac. Med. Mex.* **140**:391–398.
17. Wright, A. E., and F. Smith. 1897. On the application of the serum test to the differential diagnosis of typhoid fever and Malta fever. *BMJ* **1**:1214–1215.
18. Yagupsky, P. 1999. Detection of brucellae in blood cultures. *J. Clin. Microbiol.* **37**:3437–3442.
19. Young, E. J. 1983. Human brucellosis. *Rev. Infect. Dis.* **5**:821–824.
20. Young, E. J. 1991. Serologic diagnosis of human brucellosis: analysis of 214 cases by agglutination tests and review of the literature. *Rev. Infect. Dis.* **13**:359–372.