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Comparison of a Dipstick Assay for Detection of *Brucella*-Specific Immunoglobulin M Antibodies with Other Tests for Serodiagnosis of Human Brucellosis

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A dipstick assay for the detection of *Brucella*-specific immunoglobulin M (IgM) antibodies was evaluated by studying the serological response of 133 cultures and or serologically confirmed patients with brucellosis in its different stages along with those of 34 healthy controls. As regards patients with illness less than 3 months in duration, 93.1% tested positive by the dipstick assay, a percentage similar to that obtained in the standard serum agglutination test (SAT) (92.0%), somewhat lower than that obtained by culture (100%) and higher than that obtained by IgM enzyme-linked immunosorbent assay (ELISA) (80.5%). SAT was the most sensitive test (87.0%) for patients with illness more than 3 months in duration, followed by culture (50%), the dipstick assay (28.3%), and IgM ELISA (7.5%). The results demonstrate that the dipstick assay could well be used in the serodiagnosis of patients with acute brucellosis, as well as to identify patients with a long history of the illness. Under laboratory conditions this test has the advantage of being quick and IgM antibody-specific.

Human brucellosis is still an endemic disease in Asia, Latin America, and Mediterranean countries. In Spain, it is predominant in rural areas, with a morbidity in 1999 of 3.93 per 10^5 inhabitants (2).

Owing to the fact that the signs and symptoms of brucellosis are not pathognomonic, the clinical diagnosis should always be validated by bacteriological or serological tests. The isolation of the etiological agent is the only test that provides direct evidence for the presence of the pathogen. Taking into account that it is not always possible to achieve this and that the culture results under optimum conditions cannot be obtained in less than 4 days, it is often necessary to resort to indirect assays based on the identification of specific antibodies in the presence of *Brucella* antigens (3, 6, 22).

Some of the tests for the diagnosis of human brucellosis (10) have been described for the diagnosis of acute cases and to identify patients with a long history of the illness. To diagnose acute cases, assays have been used to detect the presence of specific immunoglobulin M (IgM) antibodies, and these include the radioimmunoassay (15), the enzyme-linked immunosorbent assay (ELISA) (4), the indirect immunofluorescence assay (8), and the 2-mercaptoethanol test (17).

Smits et al. (19) have recently developed a simple dipstick assay for the detection of specific IgM antibodies using a lipopolysaccharide (LPS) extract of *Brucella* as the antigen. This assay uses strips of nitrocellulose impregnated with LPS of *B. abortus* 1119-2 and a stabilized nonenzymatic detection reagent that consists of a monoclonal anti-human IgM antibody conjugated to colloidal dye particles (Palanyl red). The assay is performed by incubation of the test strip in a mixture of serum and detection reagent, conjugate binding making IgM antibodies reacting with the LPS epitopes visible.

The objective of this work was to evaluate the clinical utility of the dipstick assay for the serodiagnosis of patients suspected of having acute brucellosis. To this end, the dipstick assay was applied to serum samples of patients suspected to suffer from brucellosis sent to the authors' unit, and results were compared with those obtained for hemoculture, serum agglutination test (SAT), and a commercially available IgM ELISA.

MATERIALS AND METHODS

Study designs. Single serum samples collected from 167 patients were included. The laboratory diagnosis of brucellosis was performed by hemoculture and SAT, while the Rose Bengal (RB) test was used as a screening test. One hundred thirty-three patients were diagnosed with brucellosis. The diagnosis of brucellosis was based on the result of culture as the "gold standard" or on compatible clinical findings confirmed by a positive result in SAT. Patients were stratified in two groups: acute (less than 3 months of illness) and cases lasting more than 3 months from the time of the initial diagnosis of the signs and symptoms. The majority (65%) of the patients were from rural areas, and the gender (male/female) ratio was 1.8. The mean age of the patients was 42 years (range, 16 to 75 years).

Four blood samples from each patients were cultured by Bactec Plus + aerobic/F and Bactec Plus + anaerobic/F. A 10-ml volume of blood was added to the flask, and the culture was incubated at 37°C for a maximum of 6 weeks (Bactec 9240; Becton Dickinson); organisms were identified in accordance with the taxonomic criteria delineated by Weyantet al. (20).

Serology. The RB was performed as described by Morgan et al. (14) using the commercial suspension Brucelloslide (BioMérieux, Charbonières les Banes, France) as the antigen. SAT was performed according to the method described by Foz et al. (18), using an antigenic suspension prepared by Laboratorios Atom Biosystem, Barcelona, Spain. SAT was considered positive when a titer of ≥1:160 was obtained. IgM antibodies specific to *B. abortus* LPS were measured using optical density (OD) values generated by an ELISA kit (Laboratorios Vircell,

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TABLE 1. Laboratory test performance for *Brucella* according to duration of disease

Test	No. (%) of patients with a positive result		
	<3 mo of disease (n = 87)	>3 mo of disease (n = 46)	Total (n = 133)
Culture	87 (100)	23 (50)	110 (82)
RB	85 (97.7)	43 (91.5)	128 (96.2)
SAT	80 (92.0)	40 (87.0)	120 (90.2)
Dipstick	81 (93.1)	13 (28.3)	94 (70.7)
IgM ELISA[a]	70 (80.5)	3 (7.5)	73 (54.9)
IgM ELISA[b]	83 (95.4)	7 (15.2)	90 (67.7)

[a] Excluding equivocal results.
[b] Including equivocal results.

TABLE 2. Correlation of staining intensity of dipstick with result in SAT and IgM ELISA

Dipstick score	No. of patients with disease duration of:		SAT[a] median reciprocal titer (25th–75th percentile)	IgM ELISA[b] median OD (25th–75th percentile)
	<3 mo	>3 mo		
Negative	6	33	160 (160–320)	0.70 (0.60–0.90)
+1	25	9	320 (160–320)	1.02 (0.80–1.10)
+2	20	3	640 (310–640)	1.10 (1.02–1.12)
+3	17		1,280 (640–2,560)	1.12 (1.04–1.35)
+4	19	1	5,120 (1,280–5,120)	1.30 (1.10–1.80)

[a] r = 0.485; P = 0.03.
[b] r = 0.068; P = 0.01.

Granada, Spain). ELISA results were considered to be equivocal when the OD was ≥0.9 and <1.1, and positive when it was ≥1.1. The dipstick assay for the detection of *Brucella*-specific IgM antibodies was conducted by mixing 5 μl of the serum with a detection reagent (final serum dilution of 1:50) in which the LPS-impregnated nitrocellulose strip was incubated for 3 h at 37°C (7). The results were classified as negative when no coloring was observed and as positive when the antigen band showed some degree of staining, its intensity being rated from +1 to +4 using a colored reference strip (no coloring, 0; pale pink, +1; pink, +2; intense pink; +3; very intense pink, nearly red, +4).

Statistical analysis. All data were analyzed using the SPSS statistical program (Statistical Package for the Social Sciences [version 9.0]) and version 6.0 of the Center for Disease Control and Prevention's EPI-INFO program (12). The correlation between the different assays was evaluated using Pearson's correlation test. The sensitivities of the RB test, SAT, IgM ELISA, and dipstick assay were calculated using the results obtained for hemoculture-positive confirmation, or a compatible clinical assay with serology ≥1/160, as the gold standard. The following staining intensity scale was used: 0, negative; 1, pale pink; 2, pink; 3, intense pink; and 4, very intense pink, nearly red. Statistical significance was taken as $P < 0.01$.

RESULTS

Hemoculture confirmed the diagnosis of brucellosis in 110 patients, of whom 87 had acute disease and 23 had an evolution of disease longer than 3 months (Table 1). SAT was positive in 80 culture-positive patients with acute disease and in 17 culture-positive and 23 culture-negative patients with brucellosis with more than 3 months' evolution. The RB test was positive for 128 patients, of whom 85 had acute disease and 43 had disease of more than 3 months' duration. A sensitivity of 70.7% for the dipstick assay was calculated for the total group of patients. The sensitivity of the dipstick was 93.1% for patients with acute disease and 28.3% for those who had been ill for more than 3 months. The number of SAT or dipstick-positive patients with acute brucellosis was about the same, while the number of SAT-positive patients with an evolution of more than 3 months was much higher. The sensitivity of IgM ELISA was somewhat lower than that of the dipstick assay. However, the sensitivities of the two tests were about the same when equivocal ELISA results were included. SAT was the only test that gave a positive result for one patient that was finally diagnosed as having salmonellosis.

The dipstick assay gave a moderate (2+) to strong (4+) staining intensity for samples from most dipstick-positive patients with acute disease. The staining intensity was rated negative for 6 patients, +1 for 25 patients, +2 for 20 patients, +3 for 17 patients, and +4 for 19 patients (median, +2). The staining intensity for the patients with illness lasting more than 3 months was rated negative for 33 patients, +1 for 9 patients, +2 for 3 patients, and +4 for 1 patient (median is negative). Although the number of patients with disease lasting more than 3 months who were SAT positive was relatively high, the median SAT titer (1:160) for this group of patients was much reduced compared with the median SAT titer (1:640) for the group of patients with acute disease. The median OD value in ELISA was 1.1 for patients with acute disease and 0.7 for the other group. The staining intensity of the dipstick assay correlated well with the values for the SAT (r = 0.485; P = 0.03) and IgM ELISA (r = 0.068; P = 0.01) (Table 2). The greater the staining intensity was, the higher the median values of SAT and IgM ELISA were.

Discrepant results were obtained for six patients with disease lasting less than 3 months. One patient was culture positive for brucellosis but tested negative by all serological tests. Two patients tested negative by the RB test and SAT, equivocal by the IgM ELISA, and positive by the dipstick assay, and four patients tested negative by SAT, equivocal by the IgM ELISA, and positive by the RB test and dipstick.

DISCUSSION

The main *Brucella* antigen of diagnostic significance in human brucellosis is the cell surface smooth LPS (S-LPS). The bacteria, as any gram-negative in the smooth phase, have a surface covered by an outer membrane containing an LPS, which is exposed to the environment. The S-LPS is the antigenic component that plays the most important role in agglutination tests like SAT and the RB test (16). Both IgM and IgG class antibodies are active in these tests. Antibodies to the S-LPS can also be detected by a variety of tests, including ELISA, that discriminate between the class of antibody. According to these tests human brucellosis is characterized by an initial rise of IgM antibodies followed by a switch to IgG class antibodies (4, 17). Patients suffering relapse show an increase of IgG but not of IgM (9). These results indicate that at least two serological methods should be used in the diagnosis of human brucellosis to distinguish both immunoglobulin classes and to determine the stage of the infection. Sometimes patients with brucellosis have SAT titers that are positive but <1/160; therefore, a careful clinical evaluation is called for in these cases.

The diagnosis of brucellosis is made accurately when *Brucella* organisms are recovered from the blood, bone marrow, or other tissues. It is known that patients with acute brucellosis

have high rates of positive blood culture, ranging from 53.4 to 90% of patients (21).

For this reason we chose hemoculture-positive patients who did not receive a specific antibiotic treatment for *Brucella* spp., although other patients with brucellosis were not included.

The IgM dipstick assay (19) is one of the tests that have been adapted to detect IgM antibodies to the S-LPS. The assay showed a high sensitivity for patients with disease lasting less than 3 months. The detection of IgM antibodies but not IgG antibodies explains the low positivity rate of the dipstick assay and the IgM ELISA for samples collected from patients sick for more than 3 months. In contrast, the detection rates of the RB test and SAT, which detect IgM as well as IgG antibodies, were also high in samples from patients with illness lasting more than 3 months. The combined results of SAT and the dipstick assay thus provide an indication of the stage of the disease for those patients for whom the onset of clinical symptoms and signs is not known. Patients with acute illness will test positive by the dipstick assay, while those who have been ill for more than 3 months will in all likelihood test negative. However, the possibility that such patients are suffering a relapse should not be discounted.

The titers obtained in the SAT show a high degree of correlation with those obtained by testing serum dilutions in the RB test (3). In this work, three patients with acute disease confirmed by hemoculture tested negative in the RB test. Two of these patients tested positive in the dipstick assay (+1) and tested doubtful in the IgM ELISA. The SAT titers of these sera were 1:40 or less, and these results are in agreement with previous reports about the sensitivity of the RB test. False-negative SAT results may occur in patients with a recent infection (i.e., infection onset less than 10 days prior to testing in all cases) or whose serum contains blocking antibodies. Blocking antibodies are IgG or IgA that do not react at pH 7.2 but do react at pH 5.0. For this reason it is possible to get an SAT-negative and an RB-positive test. Moreover, in these cases the ELISA IgG or Coombs IgG are always positive. The "prozone" phenomenon has little practical importance. The prozone is seldom observed, and when it occurs the titer obtained is rarely higher than 1/40. (3, 16). In these cases, the dipstick assay was more sensitive than the RB test and SAT.

The sensitivity of the RB test depends on the antigenic concentration and the pH (5). Moreover, working with purified preparations of bovine immunoglobulin (IgG1, IgG2, and IgM) Díaz and Levieux (9) showed that the response of a given antibody isotype to the RB test did not depend on the class or subclass of immunoglobulin but on the commercial antigenic preparation used. These results offer an explanation for the differences of opinion found in the literature regarding the sensitivity of the RB test. For instance, Cernyseva et al. (7) found that the sensitivity of the test was 68.6%, while Diaz et al. (11) and Altwegg et al. (1) put the figure at 98.5 and 100%, respectively. If the RB test is used as a screening test, an effort must be made to use a preparation that does not give high sensitivity, thus avoiding false-negative results. The RB test can give false-negative results that can be due to an unsatisfactory antigen preparation or to a recent infection.

False-positive reactions can be due to sera from patients infected with *Yersinia enterocolitica* 0:9 or from healthy individuals who have been exposed to smooth *Brucella*, but these false-positive results do not pose any problem if other diagnostic assays (hemocultures and other serological tests) are used for confirmation.

Hypothetically speaking, the dipstick assay could replace tests based on the use of mercaptans (mercaptoethanol or dithiothreitol), because the latter could be negative in cases in which exists a great amount of agglutinating IgG antibodies resistant to the action of these agents as Marrodan et al. (13) demonstrated. In such cases as these in which SAT values do not decrease in the presence of 2-mercaptoethanol, the only way to identify the presence of IgM antibodies is by the dipstick assay or ELISA. Although the ELISA technique is considered one of the most sensitive serological test and is a useful method for monitoring antibodies in patients undergoing treatment, the lack of a standard antigen, the variations in the quality of preparations, and the use of various endpoints make difficult the interpretation of ELISA results. Our objective is to use the test routinely under laboratory conditions. However, the positivity rate of 80.5% for patients with illness lasting 3 months or less increase to a rate of 90.5% when patients with equivocal results are included. These facts could be due to the rather high background of the technique and/or related with the difficulties to prepare the antigen and to establish the cutoff points

The main objective of this work has been to compare, in a hospital service setting, the dipstick assay, a rapid and simple technique for detecting IgM antibodies, with other classical techniques (RB test, SAT, and commercial ELISA), in subjects with brucellosis. We have shown that the dipstick assay offers higher sensitivity and easier manipulation than the IgM ELISA to detect IgM antibodies to Brucella spp. and improves the interpretation of the results, establishing the cutoff points. The availability of a rapid and simple test to detect IgM antibodies to S-LPS may be useful for the diagnosis of human brucellosis. Our results demonstrate that the dipstick assay could be used as a rapid and simple alternative to the IgM ELISA for the serodiagnosis of patients with acute brucellosis.

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