Development and Evaluation of a Rapid Dipstick Assay for Serodiagnosis of Acute Human Brucellosis

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A dipstick assay for the detection of brucella-specific immunoglobulin M antibodies was evaluated with 707 sera from 247 laboratory-confirmed brucellosis patients and 342 control sera from brucellosis-free individuals. These sera were collected from six different countries. The assay was found to be highly sensitive and specific. In addition, the test is easy to use and does not require specialized training or equipment, and the components are stable without a requirement for refrigeration. All of these factors make the test ideal for developing countries and rural settings.

Brucellosis is an important but often neglected cause of morbidity in many regions of the world (1, 2, 4, 9, 17, 19, 25). The disease is most common in rural areas and among those involved in animal husbandry. Brucellosis also occurs in urban settings when animals are kept in compounds around houses and among meat-packers, dairy workers, and veterinarians. *Brucella abortus*, *Brucella suis*, and *Brucella melitensis* are the causative agents, which have, respectively, cattle, swine, and goats and sheep as their main hosts. The disease is transmitted from infected animals by direct contact with blood, fetuses and fetal membranes, uterine secretions, and aborted material or through consumption of infected, raw animal products, of which milk and milk products are the most important (26).

The treatment of chronic brucellosis is complicated and requires prolonged medication compared to that for acute brucellosis; the disease should be diagnosed and treated promptly. Typical severe acute brucellosis in its early stages cannot be diagnosed on clinical grounds along (11). Symptoms and signs are nonspecific, and several other febrile illnesses may be simulated, as for example glandular fever, influenza, malaria, and enteric infections. Also, when an unusual complication is present, brucellosis may be overlooked. Laboratory tests such as culture and serological tests including the serum agglutination test (SAT) (7, 24), the anti-human globulin test (Coombs test) (21), the complement fixation test (12), and enzyme-linked immunosorbent assay (ELISA) (5, 13, 20), therefore, are indispensable for an accurate diagnosis.

The detection of *Brucella*-specific immunoglobulin M (IgM) antibodies allows the diagnosis of patients with brucellosis at an early stage or acute disease and also may help to discriminate between patients in the early phase of brucellosis and those with chronic brucellosis. In countries where the disease is highly endemic, a large proportion of the population may have persistent *Brucella*-specific IgG antibodies. Under such conditions, the detection of specific IgM antibodies is important to make the laboratory diagnosis of brucellosis in the early phase of the disease. Specific IgM antibodies can be detected by SAT performed in the presence of either 2-mercaptoethanol (2-ME test) or dithiothreitol (SAT-DTT) (3, 10, 18, 22) and by ELISA (5, 13, 20). The SATs and ELISA can be performed only by relatively skilled personnel in well-equipped laboratories, and these tests are too elaborate for widespread application under field conditions. In situations where appropriate diagnostic facilities are lacking, a colorimetric test, with a simplified format, giving a positive or negative result could serve as a confirmatory test for human brucellosis in the acute phase of the disease. For this reason, we developed a simple dipstick assay for the detection of *Brucella*-specific IgM antibodies in human serum samples, which is evaluated in this paper.

The dipstick heat-resistant antigen was prepared from a liquid culture of *B. abortus* 1119-2 by heating washed cells at 95°C followed by removal of cell debris by centrifugation, and this preparation was then applied as a distinct line to a nitrocellulose strip (16). To obtain an internal control, an anti-human IgM antibody was applied as a coating to the nitrocellulose as a separate line (16). The coated strips were blocked with skimmed milk and dried, made to adhere to a plastic backing with double-sided tape, cut into 2.5-mm-wide sticks, and shipped with a vial of wetting agent. A nonenzymatic detection reagent was prepared by conjugation of a monoclonal anti-human IgM antibody to colloidal dye particles (palanyl red) according to a patented method (14, 15, 23). To increase stability, the stained antibody suspension was lyophilized and shipped with a rehydration reagent in a separate bottle (16). The dipstick assay is performed by incubation for 3 h of a wetted dipstick in 250 μl of reconstituted detection reagent mixed with 5 μl of a serum sample. At the end of the incubation period, the dipstick is thoroughly rinsed with tap water in order to remove excess detection reagent and air dried at

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TABLE 1. Staining intensity of dipstick assay for samples collected at different stages of the disease

Staining intensity of dipstick assay	No. of samples with indicated staining intensity at mo after onset of disease:			
	0–2	2–4	4–6	>6
Negative	8	13	35	54
1+	5	19	8	15
2+	17	14	7	5
3+	16	15	1	3
4+	27	16	1	
Total	73	77	52	77

ambient temperature. A reddish-stained antigen band indicates a positive reaction. The staining of the antigen band can be scored as 1+ through 4+ by comparison with a colored reference strip; when no coloring is observed, the test is negative. In order to assess the clinical utility of the assay, laboratories in Portugal, Russia, Spain, The Netherlands, and the United States were provided with dipsticks, test reagents, and test tubes and were asked to perform the assay according to an accompanying protocol. In the laboratories in Portugal, Russia, Spain, and The Netherlands, randomly selected serum samples from laboratory-confirmed brucellosis patients and brucellosis-free individuals were tested in order to determine the sensitivity and specificity of the assay at different stages of the disease and the results of these studies were combined. Furthermore, samples from an outbreak of brucellosis were tested in the United States, and in Yemen, a group of samples from culture-proven patients was tested.

The first study group of 150 patients included 39 patients with 71 samples from Portugal, 90 patients from Russia, 19 patients with 49 samples from Spain, and 2 patients from The Netherlands. Patients were considered laboratory-confirmed brucellosis patients based on the results of culture, SAT, and Coombs test. Thirty-nine (26%) patients had positive blood cultures, 38 of which were positive for *B. melitensis* and 1 of which was positive for *B. suis*. The control group (342 samples) included 94 patients with clinical suspicion of brucellosis but negative results in the Rose Bengal test. The remaining members of the control group were patients with the following conditions (number of patients): autoimmune disease (28), bartonellosis (*Bartonella henselae*) (2), hantavirus infection (11), hepatitis A (5), hepatitis B (7), human immunodeficiency virus infection (20), legionellosis (11), leptospirosis (8), Lyme borreliosis (20), malaria (20), meningitis (7), meningococcal meningitis (8), *Mycoplasma pneumoniae* infection (7), ochrobacteriosis (2), syphilis (20), toxoplasmosis (9), tularemia (*Francisella tularensis*) (12), *Yersinia pseudotuberculosis* II infection (1), *Yersinia enterocolitica* 03 infection (1), and *Y. enterocolitica* 09 infection (4). Forty-five serum samples from healthy donors were also included.

To calculate the sensitivity of the dipstick assay at different stages of brucellosis, the serum samples from the patients were stratified according to the duration of the disease: 73 samples collected within 2 months, 77 samples for the period of 2 to 4 months, 52 samples for the period of 4 to 6 months and 77 samples collected after >6 months of treatment (Table 1). The sensitivity of the dipstick assay was 89.0% for the samples collected within 2 months after the onset of the disease and 83.1% for the samples collected 2 to 4 months after the onset of the disease (Table 2). The sensitivity dropped to 32.6 and 29.8% for the two groups of samples collected after 4 and 6

months of treatment, respectively (Table 2). Furthermore, the staining intensity of the antigen band of the dipstick was moderate (2+) to strong (4+) for most of the positive samples collected early in the disease (Table 1). The percentages of the four groups that stained as >1+ declined with the duration of treatment: 82.2, 58.4, 17.3, and 10.3%, respectively (Table 1). Compared with that of the SAT, the sensitivity of the dipstick assay was higher for the samples collected during the first 4 months of the disease but lower for the samples collected after the fourth month (Table 2). The sensitivity of the dipstick assay for the samples collected during the first 4 months also was higher than or equal to that of the Coombs test (Table 2). The sensitivity of the dipstick assay was higher than the sensitivity of the SAT performed in the presence of reducing agent, an assay often used to assess the presence of specific IgM antibodies, for all four groups of samples. The difference in sensitivity between these two assays was largest for the samples collected 2 to 4 months after the onset of the disease. Samples collected from 5 of 89 patients during the first 6 months of the disease were negative in the dipstick assay. These samples were also negative in the 2-ME test. The clinical symptoms of these five patients were consistent with chronic brucellosis rather than acute or recent-onset brucellosis; the final diagnosis was *Brucella* arthritis, spondylitis, and neurobrucellosis for one patient each, and *Brucella* was isolated from bone marrow from two patients. Only 4 of 297 samples from the noncase patients gave a positive result in the dipstick assay, giving a specificity of 98.6%. These four patients included one suspected brucellosis patient with a negative result in the Rose Bengal test, one patient with syphilis, and two patients with yersiniosis. The staining intensity of all four samples was rated 2+. None of the 45 blood bank sera gave a positive result.

Of the case patients from Portugal, Russia, and Spain, 39 patients had a positive blood culture. Serum samples from 36 (92.3%) of these 39 blood culture-positive patients gave a positive result in the dipstick assay. Of the patients with a positive result in the dipstick assay, one was culture positive for *B. suis* and the others were positive for *B. melitensis*. To demonstrate the reactivity of the dipstick for patients infected with *B. abortus*, a group of single serum samples from 60 culture-proven patients from Yemen was tested. Of these patients, 42 had a positive culture for *B. abortus* and 18 were positive for *B. melitensis*. The sensitivity of the dipstick assay for these two groups of patients was 95.2 and 83.3%, respectively. The majority (53 and 80%, respectively) of the dipstick-positive sam-

TABLE 2. Sensitivity of dipstick assay and other serological tests in relation to duration of the disease

Test[a]	Sensitivity (%) at mo after onset of disease:			
	0–2	2–4	4–6	>6
Dipstick assay	89.0	83.1	32.6	29.8
SAT	79.5	64.4	76.9	50.6
SAT-DTT and 2-ME[b]	67.1	19.1	13.5	6.0
Coombs test	39.7	88.1	92.3	81.8

[a] The dipstick assay was considered positive when a staining intensity of >1+ was observed. The SAT was considered positive when a titer of ≥1:160 was obtained. The 2-ME test and the SAT-DTT were considered positive when a ≥4-fold reduction in titer compared with that of the SAT was observed. The Coombs test was considered positive when a ≥4-fold increase in titer was measured.

[b] The SAT-DTT was performed on the samples from Spain. The 2-ME test was performed on the samples from Portugal. The SAT-DTT or the 2-ME test was not done for the samples from Russia and The Netherlands.

TABLE 3. Dipstick results and sensitivity for serum samples collected from brucellosis patients during a *B. suis* slaughterhouse outbreak in the United States (1976)

Sample group (*n*)	No. of samples with the indicated staining intensity:					Sensitivity (%)
	0	1+	2+	3+	4+	
Initial (37)		1	4	13	19	100
12 mo (36)	14	14	4	4		61.1

ples of these two groups gave a moderate to strong staining intensity.

To validate the dipstick assay further and to test the reactivity of the dipstick assay for patients with a *B. suis* infection, the assay was applied on 93 paired serum samples derived from 37 laboratory-confirmed brucellosis patients infected with *B. suis* during a localized outbreak in a slaughterhouse in the United States during 1976. All initial serum samples tested positive in the dipstick, giving a sensitivity of 100% (Table 3). Of the samples taken 12 months on average after the first sample, 61% still tested positive, but while the staining intensity of the initial samples was ≥2+ for all but one sample, most of the subsequent samples gave a 1+ staining intensity.

The results of the present study show that the dipstick assay is highly sensitive and specific for the serodiagnosis of human brucellosis in the early phase of the disease. For the combined group of samples from Portugal, Russia, Spain, and The Netherlands, the sensitivity was 89.0% for samples collected during the first 2 months of the disease and 83.1% for samples collected 2 to 4 months after the onset of the disease. The sensitivity for the group of samples from the United States collected early in the disease during an outbreak of brucellosis was even higher (100%). Infection with *B. melitensis* was the most common cause of disease among the first study group. All patients in the outbreak were infected with *B. suis*. The somewhat lower sensitivity of the dipstick assay calculated for the results of the first study, however, most likely was not related to the difference in causative agent. Some of the patients with a negative result in the dipstick assay from the first study group likely suffered from chronic disease despite the reported recent onset of the disease, and this may well have accounted for the lower sensitivity. The dipstick assay performed equally well for the confirmation of suspected brucellosis patients with *B. abortus*, *B. melitensis*, or *B. suis* infections. The sensitivity was high irrespective of whether it was calculated based on the results obtained for patients confirmed by serological methods or by culture. The sensitivity was 93.5% when only blood culture-proven brucellosis patients were considered.

The specificity of the dipstick assay was calculated to be 98.6%. The selectivity for sera of patients with *Y. enterocolitica* or cholera infection needs further investigation, as these organisms share antigenic structures with brucellas (6, 8). However, the symptoms of patients with yersiniosis or cholera are distinct from those of patients with brucellosis.

The high sensitivity and specificity of the dipstick assay for samples collected in the acute phase of the disease demonstrated that the assay is highly suitable for use in serodiagnosis of patients with acute disease. Compared with SAT-DTT or the 2-ME test, the dipstick assay has a higher sensitivity and is easier to use: the dipstick assay requires only a few minutes of handling time to perform, does not need special equipment or electricity, and can be performed by modestly trained personnel with a minimum of instructions. The dipstick assay also is easier to apply than ELISA. The dipstick assay is suited for use in the field and in laboratories that are not equipped to perform the more complicated tests. The assay also has potential to replace the other methods for distinguishing patients with acute brucellosis from those with a chronic infection or with persisting IgG antibodies due to a previous infection. It may be noted, though, that consistent with the results of ELISA (7, 11) our results show that specific IgM antibodies may remain detectable for several months and sometimes even much longer after the onset of the disease. However, while most serum samples collected early in the disease gave a ≥2+ staining, most of the sera collected later, 4 to 6 months after treatment had been initiated, tested as 1+.

The five laboratories participating in the study found the test easy to perform and were satisfied with the result. It was also noted that the development of color on the dipstick could be maintained as a permanent record in a folder or attached to the patient record.

In conclusion, the dipstick assay described here is an easy-to-perform method for the quick serodiagnosis of acute human brucellosis. Due to its robustness and simplicity, the assay is highly suitable for application under field conditions. Ideally, application of two dipsticks, one for the detection of specific IgM antibodies and another for the detection of specific IgG antibodies, would be needed to cover the possibility of both acute or recent and chronic brucellosis. The development of an IgG-specific rapid test for brucellosis is now in progress. A further prospective study will be required to demonstrate the clinical utility of the assay and to calculate the sensitivity, specificity, and predictive value for patients living in an area where brucellosis is endemic.

REFERENCES

1. **Abo-Shehada, M., J. S. Odeh, M. Abu-Essud, and N. Abuharfeil.** 1996. Seroprevalence of brucellosis among high risk people in Northern Jordan. Int. J. Epidemiol. **25:**450–454.
2. **Al-Eissa, Y., F. Al-Zamil, M. Al-Mugeiren, S. Al-Rasheed, A. Al-Sanie, and A. Al-Mazyad.** 1991. Childhood brucellosis: a deceptive infectious disease. Scand. J. Infect. Dis. **23:**129–133.
3. **Anderson, R. K., R. Jenness, H. P. Brumfield, and P. Gough.** 1964. Brucella-agglutinating antibodies: relation of mercaptoethanol stability to complement fixation. Science **143:**1334–1335.
4. **Araj, G. F., and R. A. Azzam.** 1996. Seroprevalence of brucella antibodies among persons in high-risk occupation in Lebanon. Epidemiol. Infect. **117:**281–288.
5. **Ariza, J., T. Pellicer, R. Pallarés, and F. Gudiol.** 1992. Specific antibody profile in human brucellosis. Clin. Infect. Dis. **14:**131–140.
6. **Avhonen, P., and K. Sievers.** 1969. *Yersinia enterocolitica* infection associated with *Brucella* agglutinins. Clinical feature of 24 patients. Acta Med. Scand. **185:**121–125.
7. **Buchanan, T. M., C. R. Sulzer, M. K. Frix, and R. A. Feldman.** 1974. Brucellosis in the United States, 1960–1972. An abattoir-associated disease. Medicine **53:**415–425.
8. **Dajani, Y. F., A. Aziz Masoud, and H. F. Barakat.** 1989. Epidemiology and diagnosis of human brucellosis in Jordan. J. Trop. Med. Hyg. **92:**209–214.
9. **Díaz, R., E. Maraví-Poma, S. Fernández, S. García-Merlo, and A. A. Rivero-Puento.** 1982. Brucellosis: estudio de 222 casos. Parte IV: diagnóstico de la brucellosis humana. Rev. Clin. Esp. **166:**107–110.
10. **Díaz, R., I. Moriyón, C. Gamazo, B. Alonso-Urmeneta, and M. de la Vinda.** 1991. Laboratory diagnosis of human brucellosis, p. 93–107. *In* M. Plommet (ed.), Prevention and control of brucellosis in the Mediterranean countries. Pudoc Scientific Publishers, Wageningen, The Netherlands.
11. **Caroff, M., M. B. Bundle, J. W. Perry, J. W. Cherwonogrodzky, and J. R. Duncan.** 1984. Antigen S-type lipopolysaccharide of *Brucella abortus* 1119-3. Infect. Immun. **46:**383–388.
12. **Foz, A., and L. Arcalis.** 1953. Die Komplementbindungs-Reaktion in der diagnosis der menschlichen brucellosis. Z. Hyg. **136:**55–66.
13. **Gazapo, E., J. Gonzales Lahoz, J. L. Subiza, M. Baquera, J. Gil, and E. G. de la Concha.** 1989. Changes in IgM and IgG antibody concentrations in brucellosis over time: importance for diagnosis and follow-up. J. Infect. Dis. **159:**219–225.
14. **Gribnau, T., F. Roeles, J. van der Biezen, J. Leuvering, and A. Schuurs.** 1982.

The application of colloidal dye particles as label in immunoassays: disperse(d) dye immunoassay ("DIA"), p. 411–424. *In* T. C. J. Gribnau, J. Visser, and R. J. F. Nivard (ed.), Affinity chromatography and related techniques. Elsevier, Amsterdam, The Netherlands.

15. **Gribnau, T., A. van Someren, and F. van Dinther.** 1983. DIA-disperse dye immunoassay, p. 275–280. *In* I. M. Chaiken, M. Wilchek, and I. Parikh (ed.), Affinity chromatography and biological recognition. Academic Press, Inc., Orlando, Fla.

16. **Gussenhoven, G. C., M. A. W. G. van der Hoorn, M. G. A. Goris, W. J. Terpstra, R. A. Hartskeerl, B. W. Mol, C. W. van Ingen, and H. L. Smits.** 1997. LEPTO Dipstick, a dipstick assay for the detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. J. Clin. Microbiol. **35:**92–97.

17. **Idris, M. A., M. Maiwald, K. N. El-Mauly, and A. Ruppel.** 1993. Human brucellosis in Dhofar, Sultanate of Oman. J. Trop. Med. Hyg. **96:**46–50.

18. **Kerr, W. R., W. J. Mccaughey, J. D. Coghlan, D. J. H. Payne, R. A. Quaife, L. Robertson, and D. Farrell.** 1968. Techniques and interpretation in the serodiagnosis of brucellosis in man. J. Med. Microbiol. **1:**181–193.

19. **Kiel, F. W., and M. Y. Khan.** 1987. Analysis of 506 consecutive positive serological tests. J. Clin. Microbiol. **25:**1384–1387.

20. **Lulu, A. R., 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.

21. **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.

22. **Reddin, L., R. K. Anderson, R. Jenness, and W. W. Spink.** 1965. Significance of 7S and macroglobulin brucella agglutinins in human brucellosis. N. Engl. J. Med. **272:**1263–1268.

23. **Snowden, K., and M. Hommel.** 1991. Antigen detection immunoassay using dipsticks and colloidal dyes. J. Immunol. Methods **140:**57–65.

24. **Spink, W. W., N. B. McCullough, L. M. Hutchings, and C. K. Mingle.** 1954. A standardized antigen and agglutination technique for human brucellosis. Am. J. Clin. Pathol. **24:**496–498.

25. **Veerman, J., M. A. Basahi, and M. Al Qadasi.** 1995. Human brucellosis. An old enemy or a new threat? Practitioner (East Mediterr. Ed.) **15:**687–689.

26. **Young, E. J.** 1994. An overview of human brucellosis. Clin. Infect. Dis. **21:**283–290.