Evaluation of an Immunocapture-Agglutination Test (Brucellacapt) for Serodiagnosis of Human Brucellosis

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We evaluated the validity and the usefulness of a new test for the diagnosis of human brucellosis based on an immunocapture-agglutination technique. A total of 315 sera from 82 patients with a diagnosis of brucellosis, 157 sera from patients in whom brucellosis was suspected but not confirmed, and 412 sera from people living in rural areas with endemic brucellosis were studied. The seroagglutination test (SAT), Coombs anti-Brucella test, and Brucellacapt test were evaluated. All the initial sera from the 82 patients proved to be positive in Brucellacapt and Coombs tests, while only 75 (91.4%) were positive in the SAT. If a ≥1/160 diagnostic threshold titer was defined for the Brucellacapt test, Coombs test, and SAT, the sensitivities were 95.1, 91.5, and 65.8%, respectively. Taking the same diagnostic threshold titer for the 157 sera from the unconfirmed but suspected patients, the specificities of the Brucellacapt, Coombs, and SAT were 81.5, 96.2, and 100%, respectively; for the 412 control sera, the specificities were 99.0, 99.8, and 100%. The diagnostic efficiency (area below the receiver operating characteristic curve) of Brucellacapt was 0.987852 (95% confidence interval [CI], 0.95109 to 0.99286), very similar to the diagnostic efficiency of the Coombs test (0.9761; 95% CI, 0.94781 to 0.99146) and higher than that of SAT (0.91013; 95% CI, 0.86649 to 0.94317). The results of the Brucellacapt test were compared with those of the Coombs test (correlation coefficient, 0.956; P = 0.000) and SAT (correlation coefficient, 0.866; P = 0.000). The study shows very good correlation between the Brucellacapt and Coombs tests, with a high concordance between titers obtained in the two tests. Nevertheless, lower correlation and concordance were found between the Brucellacapt and Coombs tests when the results for titers of ≥1/160 were compared (0.692; P = 0.000). In acute brucellosis, the Brucellacapt and Coombs tests render positive titers of ≥1/160. When the titers are lower, they increase significantly in the following 30 days, despite the evolution of SAT titers. In contrast, Brucellacapt and Coombs titers are always high (≥1/640) in brucellosis with long evolution, whether SAT titers are higher or lower than 1/160.

Brucellosis is a zoonosis caused by bacteria of the genus Brucella, which affect both humans and animals such as cattle, sheep, goats, and swine. This disease is worldwide, with areas of high endemicity such as the Mediterranean, Middle East, Latin America, and Asia (11, 26). The incidence in humans ranges widely between different regions, with values of up to 200 cases per 100,000 population.

Human brucellosis has a great variety of clinical manifestations, making it difficult to diagnose clinically. Therefore, the diagnosis must be confirmed by isolation of Brucella, mostly from blood culture or by the detection of an immune response to its antigens. The diagnosis of brucellosis based exclusively on Brucella isolation presents several drawbacks. The slow growth of Brucella in primocultures may delay diagnosis for more than 7 days (5, 31, 37). Also, blood culture sensitivity is often low, ranging from 50 to 90% depending on disease stage, Brucella species, culture medium, quantity of circulating bacteria, and the blood culture technique employed (23, 37). Hence, serological tests play a major role in cases when the disease cannot be detected by blood culture. However, the interpretation of these tests is often difficult, particularly in patients with chronic brucellosis, in reinfections and relapses, and in areas of endemicity, where a high portion of the population have antibodies against brucellosis.

Many serological tests have been used for the diagnosis of human brucellosis. The most commonly used tests are the serum agglutination test (SAT), the Coombs anti-Brucella test, the Rose Bengal test, and complement fixation. During the last decade, radioimmunoassay (24, 28) and enzyme immunoassay (6, 21, 34) tests have also been used. These present technical difficulties since they require skilled personnel and high-cost material. Also, interpretation of enzyme immunoassay results is difficult due to the variability of antigens and technical procedures employed.

Among the techniques used for the diagnosis of human brucellosis, SAT and the Coombs test are most often used, and their performance in disease diagnosis and during disease evolution has been studied thoroughly (38). However, their evaluation is sometimes uncertain, and the interpretation of SAT titers of ≤1/160 is problematic in areas of endemicity, since low SAT titers may be present in healthy people who previously suffered the disease (6), in patients during the first stage of the infection (19, 38), and in patients suffering chronic brucellosis or a relapse (29). Diagnosis of a relapse is particularly difficult and is most often based on the presence of high titers in the Coombs test (6, 29). However, this is a long and technically difficult test, requiring skilled personnel, and so it is not routinely performed in many clinical laboratories.

The convenience of using Brucellacapt, a new serological
test for the diagnosis of human brucellosis based on immuno-capture-agglutination of total anti-Brucella antibodies, is discussed in the present paper.

**MATERIALS AND METHODS**

**Clinical material.** A total of 884 sera from different groups of patients were studied. The first 315 sera were from 82 patients with a diagnosis of brucellosis (78 with acute brucellosis and 4 with chronic brucellosis). Of the 82 patients, 17 were women (20.7%), with an average age of 43.0 years, (standard deviation [SD] 7.8 years, range, 24 to 68 years) and 65 were men (79.3%), with an average age of 33.3 years (SD, 15.1; range, 7 to 71 years). Brucellosis was diagnosed on the basis of clinical evidence, and the diagnosis was confirmed by isolation of *Brucella* in blood culture, a SAT titer of ≥1/160, or a fourfold rise in SAT or Coombs test titers between two samples collected within 15 to 30 days of each other. At least one blood culture was carried out for every patient, with positive results in 61 cases. *Brucella melitensis* biovar 3 was isolated in all cases. The four patients with chronic brucellosis presented clinical manifestations for more than 6 months, and in three of them *B. melitensis* biovar 3 was isolated. Of the 82 patients, 50 had a clinical and serological follow-up during the first month after diagnosis and every 2 months thereafter for at least 9 months after the initiation of antibiotic treatment. Six of the patients showed a relapse or reinfection during the follow-up, evidenced by the reappearance of symptoms after the end of treatment. In three of the 15 *B. melitensis* biovar 3 was isolated. Of these six patients, three experienced the relapse or reinfection 2 months after the end of treatment and three experienced it between 6 and 12 months after treatment.

To perform validation studies of serological tests, the first serum sample obtained from each patient in the course of the current disease was considered the initial serum sample. Sera obtained during clinical and serological follow-up were considered evolutive sera.

A further 157 sera from patients in whom brucellosis was suspected but could not be confirmed were also studied. A positive Coombs test (≥1/20) and SAT titers of <1/160 were obtained for 84 of these sera.

A total of 412 sera from people living in rural areas of a region with endemic brucellosis (Castilla y León, Spain) were included as control group. They were randomly selected among people attending outpatient clinics. None of the patients was diagnosed with brucellosis.

**Methods.** SAT, the Coombs anti-Brucella test, and Brucellacapt were performed for each serum sample. All samples from a given patient were processed simultaneously. The SAT and Coombs test were performed in tube by a double-dilution method from an initial 1/20 dilution, using a commercial *B. abortus* antigen (Linear Chemicals). SAT reactions were read after a 24-h incubation at 37°C. The highest serum dilution showing >50% agglutination was considered the agglutinating titer. The Coombs test was carried out with the SAT tubes by washing three times with phosphate-buffered saline (pH 7.2) by centrifugation at 3,000 × g for 20 min. After the last wash, the bacteria were suspended in 1 ml of phosphate-buffered saline, and 0.05 ml of previously standardized anti-human immunoglobulin (Sanofi Pasteur) was added to each tube. The tube contents were mixed and incubated at 37°C for 24 h. The results were read as described above for SAT.

The Brucellacapt test (VirCell SL) was performed as specified by the manufacturer. Briefly, 0.050 ml samples of serum dilutions were added to wells of a U-bottom microtiter plate coated with anti-total human immunoglobulin. Then 0.050 ml of an antigen suspension (colored *B. melitensis* bacteria killed by formaldehyde treatment) was added to all the wells. The plates were sealed with adhesive tape and incubated at 37°C for 24 h in a dark humid chamber. Positive reactions show agglutination over the bottom of the well. Negative reactions are indicated by a pellet at the center of the bottom of the well.

**Statistical analysis.** Data were analyzed with the help of SPSS 8.0 for Windows and Two-by-two analyzer 1.0 programs. The diagnostic validity of the Brucellacapt and Coombs tests was evaluated in the 82 initial sera from patients with brucellosis, 157 sera from the unconfirmed but suspected patients, and 412 from control sera. The results from Brucellacapt were compared with those from the Coombs test or SAT in all 884 sera studied.

In both studies, sensitivity, specificity, and likelihood ratio for positive and negative results were calculated. The 95% confidence intervals (CI) were calculated for sensitivity and specificity by the methods of Fleiss (17) and Diamond (13).

The likelihood ratio (LR) for a positive result is given by the ratio between the probability of a positive result in positives and negatives according to the "gold standard." LR values of >10 for positives are considered conclusive. The LR for a negative result is given by the ratio between the probability of a negative result in positives and negatives according to the "gold standard." LR values of <0.1 for negatives are considered conclusive; values between 0.1 and 0.2 would produce moderate changes in the pretest probability. LR 95% CI were calculated by the methods of Koopman (25), Miettinen and Nurminen (27), and Gart and Nam (20).

Spearman’s correlation coefficient was calculated for the correlation analysis between titers obtained with Brucellacapt and SAT or the Coombs test. The results of sensitivity and specificity obtained with Brucellacapt, Coombs test, and SAT titers for different cutoff points were graphically represented by a curve of diagnostic efficiency (receiver operating characteristic curve or ROC curve). The area under the curve was calculated with a corresponding CI. An SPSS macro developed by Doménech and Bonillo (16) was used for this analysis.

**RESULTS**

**Usefulness of the Brucellacapt test for the diagnosis of human brucellosis.** To study the validity of Brucellacapt for the diagnosis of human brucellosis, initial sera from 82 brucellosis patients, 157 sera from the unconfirmed but suspected patients, and 412 sera from the control group were included.

All the initial sera from the 82 patients gave titers of ≥1/20 in the Brucellacapt and Coombs tests, while only 75 (91.4%) were positive in the SAT at the same titer (Table 1). Of 7 SAT-negative sera, 5 were from patients with acute brucellosis (4 had positive blood cultures and 1 seroconverted 15 days later) and 2 were from patients with chronic brucellosis (1 with a positive blood culture). In the unconfirmed but suspected patients, 86 of the 157 sera studied (54.8%) gave titers of between 1/20 and 1/640 in the Brucellacapt test, 84 (53.5%) were positive in the Coombs test (all with titers of <1/320), and 18 (11.4%) were positive in the SAT (all titers were <1/80). In the control group, 15 of 412 sera (3.6%) were Brucellacapt
positive (all with titers of $\geq 1/1280$) and 5 (1.2%) were Coombs positive (all with titers of $\geq 1/160$). Two sera (0.48%) from the control group were SAT positive (both with $1/120$ titers).

Based on the results obtained from the 82 initial sera, if a 1/160 diagnostic threshold titer was defined for Brucellacapt, only 4 initial sera were negative (95.1% sensitivity) (Tables 1 and 2). These sera had Brucellacapt titers of 1/20 (1 serum sample) and 1/80 (3 sera). In the Coombs test, 7 initial sera showed titers of $< 1/160$ (91.5% sensitivity). All initial sera with Brucellacapt and Coombs titers of $\leq 1/160$ were from patients with acute brucellosis and evolution periods of less than 30 days. When these tests were repeated with sera taken between 15 and 30 days later, they gave positive results with titers of $\geq 1/1280$ in all the postrelapse sera. Of the 28 SAT-positive patients, 20 (17 with acute brucellosis, 2 with chronic brucellosis, and 1 with a relapse) had positive blood cultures. Four patients with acute brucellosis had Brucellacapt titers of $\leq 1/80$ in the initial sera and the same titer in the follow-up sera, collected 2 months later.

Using 1/160 as the diagnostic threshold titer, in the group of 157 sera from unconfirmed but suspected patients, 29 were positive in the Brucellacapt (specificity, 81.5%) and 6 were positive in the Coombs test (specificity, 96.2%; Table 2). If 1/320 is taken as the positivity threshold, Brucellacapt specificity rose to 97.4% and Coombs specificity rose to 100%. Using the same diagnostic threshold titer (1/160) for the 412 control sera, 4 sera were Brucellacapt positive (99.0% specificity) and 1 was Coombs positive (99.8% specificity) (data not shown). No control sera had SAT titers of $\geq 1/160$.

LR values positive for titers of $\geq 1/320$ in Brucellacapt were always above 10 (Table 2). The same was true in the Coombs test for titers of $\geq 1/160$. However, for SAT, these positive LR values were found only for titers of $\geq 1/40$. In contrast, negative LR values were below 0.1 for titers of $\leq 1/320$ in Brucellacapt, $\leq 1/160$ in the Coombs test, and $\leq 1/40$ in the SAT test.

The diagnostic efficiency of the Brucellacapt and Coombs tests and SAT has been studied (Fig. 1). The area below the ROC curve for Brucellacapt was 0.987852 (95% CI, 0.95109 to 0.99286), that for the Coombs test was 0.9761 (95% CI, 0.94781 to 0.99146), and that for the SAT was 0.91013 (95% CI, 0.86649 to 0.94317).

The serological behavior of four patients with chronic brucellosis and six patients who suffered relapse or reinfection was studied during the follow-up period. Three of the four patients with chronic brucellosis had positive blood cultures (Table 3). One of these three patients was SAT negative, one had a SAT titer of 1/40, and one had a SAT titer of 1/160. The Coombs test results obtained gave titers of 1/640 (two patients) and 1/1280 (one patient). Brucellacapt gave titers of 1/1280 (one patient) and 1/2,560 (two patients). The chronic brucellosis patient with a negative blood culture was SAT negative and had Coombs and Brucellacapt titers of 1/5,120 and 1/20,480, respectively (Table 3). Three of the six patients with relapse or reinfection during the follow-up period had positive blood cultures (Table 4). When their serological results before and after the relapse were compared, a rise in antibody titers was observed in all cases, although SAT titers were never $> 1/640$. Coombs titers were always $\geq 1/640$ in all the postrelapse sera. In these sera, Brucellacapt titers exceeded Coombs titers in all cases and were always $\geq 1/1280$.

Based on the data obtained from all 884 sera analyzed, the results of the Brucellacapt test were compared with those of the Coombs test (Table 5) and SAT (Table 6). Of 503 negative sera in the Coombs test, 29 were Brucellacapt positive (Table 5). On the other hand, 5 of the 381 Coombs-positive sera were Brucellacapt negative (correlation coefficient, 0.956; $P = 0.000$). Of 606 SAT-negative sera, 127 were Brucellacapt positive (Table 6), and none of the 278 SAT-positive sera was Brucellacapt negative (correlation coefficient, 0.866; $P = 0.000$). When only sera with titers of $\geq 1/160$ in the Brucella-
When capt and Coombs tests and SAT were considered, the correlation coefficient between the Brucellacapt and Coombs tests fell to 0.692 (P = 0.000), while that between the Brucellacapt test and SAT fell to 0.448 (P = 0.000) (Tables 5 and 6).

**DISCUSSION**

The results from the present study show a high sensitivity and specificity of Brucellacapt for the diagnosis of human brucellosis both in the first stages of the disease and in cases with long evolution as well as in relapses and reinfections. All the initial sera from patients with brucellosis included in the study had Brucellacapt and Coombs titers of ≥1/20, while only 91.4% of them were SAT positive. However, problems in the interpretation arose with the use of a 1/20 diagnostic titer, especially in areas of endemicity, where the prevalence of anti-Brucella antibodies is high due to previous episodes of brucellosis or exposure to infected animals in a high proportion of the population (1, 17, 32). In fact, positive sera were found in all tests in the control group, which was made up of individuals from a brucellosis-endemic area. Moreover, low anti-Brucella titers are found in patients who have been infected with Yersinia enterocolitica O:9, Pseudomonas maltophilia, and some Salmonella serotypes (7, 12, 15).

The definition of a diagnostic titer, indicative of an active infection, has not been possible in human brucellosis, even in tests such as the Coombs test and SAT, which have been in use for a long time (14). Most authors (8, 10, 31, 36, 38) consider a SAT titer of ≥1/160 to be indicative of active brucellosis. However, active brucellosis cannot be excluded in patients with lower SAT titers, especially during the first stage of the infection (6), in chronic brucellosis (4), and in relapses (29). In the present study, nearly 35% of the initial sera from infected patients showed SAT titers of <1/160. This implies a serious limitation for disease diagnosis, especially since prompt treatment is very important for a good prognosis (33). Our study shows that titers of ≥1/320 in Brucellacapt, ≥1/160 in the

<table>
<thead>
<tr>
<th>Patient</th>
<th>Blood culture</th>
<th>Titer* in:</th>
<th>Symptoms</th>
<th>Duration of symptoms (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcr1</td>
<td>Positive</td>
<td>40</td>
<td>1,280</td>
<td>Fever, sacroiliac inflammation</td>
</tr>
<tr>
<td>Bcr2</td>
<td>Positive</td>
<td>0</td>
<td>2,560</td>
<td>Fever, arthritis</td>
</tr>
<tr>
<td>Bcr3</td>
<td>Negative</td>
<td>0</td>
<td>20,480</td>
<td>Fever, arthralgia</td>
</tr>
<tr>
<td>Bcr4</td>
<td>Positive</td>
<td>160</td>
<td>2,560</td>
<td>Fever, sacroiliac inflam</td>
</tr>
</tbody>
</table>

* Inverse titer.
and IgM antibodies appear promptly after titer allows brucellosis to be diagnosed. In this sense, both IgG seroconversion accompanied by a significant rise in antibody enzyme immunoassay techniques (3, 4, 6, 34). In these cases, patients with brucellosis do not have detectable levels of specific antibodies cannot be detected in some brucellosis patients. According to recent studies by Smits et al. (35), 11% of patients with brucellosis do not have detectable levels of specific IgM by immunoblot analysis. Similar results were found with enzyme immunoassay techniques (3, 4, 6, 34). In these cases, seroconversion accompanied by a significant rise in antibody titer allows brucellosis to be diagnosed. In this sense, both IgG and IgM antibodies appear promptly after Brucella infection (4, 6, 21), and their concentration rises during the following days (21). Similar results were found in our study, where low titers in the Brucellacapt or Coombs test increased during the following 15 or 30 days whether SAT titers rose or not. In fact, in four patients with acute brucellosis, neither the initial serum sample nor samples collected during follow-up showed SAT titers of ≥1/160, probably due to the early initiation of therapy. In these cases, the Brucellacapt and Coombs tests gave titers between two- and fourfold higher than SAT for initial sera and between four- and eightfold higher for sera collected during the follow-up period.

Most studies show that IgM antibodies are not useful for the diagnosis of chronic brucellosis and relapses (3, 4, 35). In these cases, SAT titers are often <1/160 (2, 6, 21). However, the Brucellacapt and Coombs tests behave in a different way from that described for acute brucellosis. All patients with chronic brucellosis or relapses had Brucellacapt titers of ≥1/1280 and Coombs titers of ≥1/640. Even in two patients with relapse and two patients with chronic brucellosis, whose sera had SAT titers of <1/160, the Coombs and Brucellacapt titers were between 4- and 32-fold higher. These findings agree with those described by Foz et al. (18) using the Coombs test to detect chronic brucellosis.

Moreover, Pellicer et al. (29) observed that patients suffering a relapse had a rise in Coombs titers and specific IgG levels detected by enzyme-linked immunosorbent assay. This increase was also found in the present study with both the Brucellacapt and Coombs tests in all cases.

Finally, the study shows very good correlation between the Brucellacapt and Coombs tests, with a good concordance between titers obtained by both tests even when only titers higher than 1/160 were compared. Similar results were found by Gomez et al. (22) with sera from brucellosis patients or unconfirmed but suspected brucellosis patients. In fact, our result show that the diagnostic efficiency of Brucellacapt is equal to that of the Coombs test, as their ROC curves show. Nevertheless, a lower correlation and concordance were found between Brucellacapt and SAT.

Briefly, our study shows that the Brucellacapt and Coombs tests have very similar performances in the diagnosis of human brucellosis. Brucellacapt is more sensitive and usually shows higher titers than the Coombs test, although its specificity decreases slightly when titers less than 1/320 are used as the diagnostic threshold. In acute brucellosis, both tests have positive titers of ≥1/160. When the titers are lower, they increase significantly in the following 30 days, whether or not the SAT titers rise. In contrast, the Brucellacapt and Coombs titers are always high (≥1/640) in patients with brucellosis of long evolution, whether the SAT titers are higher or lower than 1/160.

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**TABLE 4. Antibody titer in serum samples taken before and after relapse or reinfection in the six affected brucellosis patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Blood culture</th>
<th>Titer* in:</th>
<th>SAT</th>
<th>Coombs test</th>
<th>Brucellacapt test</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Before relapse</td>
<td>After relapse</td>
<td>Before relapse</td>
<td>After relapse</td>
</tr>
<tr>
<td>Rec1</td>
<td>Positive</td>
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<td>20</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
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<td>Positive</td>
<td>80</td>
<td>640</td>
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<td>40</td>
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<td>320</td>
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</tr>
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<td>Negative</td>
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<td>320</td>
<td>320</td>
<td>1,280</td>
</tr>
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<td>640</td>
<td>640</td>
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</table>

* Inverse titer.

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**TABLE 5. Relation between the titers of the Brucellacapt and Coombs anti-Brucella tests**

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<tr>
<th>Brucellacapt titer</th>
<th>No. of samples with Coombs' anti-Brucella test titer of:</th>
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<th>160</th>
<th>320</th>
<th>640</th>
<th>1,280</th>
<th>2,560</th>
<th>5,120</th>
<th>Total</th>
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<td>10</td>
</tr>
</tbody>
</table>

Total 606 884

* Correlation coefficient for all results was 0.866 (P = 0.000). Correlation coefficient for titers of ≥1/160 (italic numbers) was 0.448 (P = 0.000).

b Inverse titer.
REFERENCES


