BRUCELLACAPT vs. CLASSICAL TESTS IN THE SEROLOGICAL DIAGNOSIS AND MANAGEMENT OF HUMAN BRUCELLOSIS

Aurora Casanova¹, Javier Ariza², Manuel Rubio³, Cristina Masuet⁴ and Ramón Díaz³

¹Department of Microbiology. Hospital Universitari de Bellvitge. Universidad de Barcelona. IDIBELL. L’Hospitalet de Llobregat. Barcelona. Spain
²Department of Infectious Diseases. Hospital Universitari de Bellvitge. Universidad de Barcelona. IDIBELL. L’Hospitalet de Llobregat. Barcelona. Spain
³Department of Microbiology. Clinica Universitaria de Navarra. Pamplona, Spain
⁴Epidemiology Unit. Hospital Universitari de Bellvitge. IDIBELL. L’Hospitalet de Llobregat. Barcelona. Spain

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**Corresponding author:** A. Casanova. Department of Microbiology. Hospital Universitari de Bellvitge. c/Feixa Llarga s/n. 08907, L’Hospitalet de Llobregat, Barcelona, Spain

e-mail: acasanova@bellvitgehospital.cat
ABSTRACT

BrucellaCapt is an immunocapture agglutination test suggested as a possible substitute for the Coombs test in the diagnosis of human brucellosis. Here it is compared with classical tests in 321 samples from 48 patients with brucellosis (6.9 ± 1.7 samples per patient), 20 of them with focal disease and 9 relapse episodes (mean follow-up: 18 months). BrucellaCapt was used according to the manufacturer’s instructions, while we also applied a variant of BrucellaCapt in which the microtiter plates were not coated with anti-total human immunoglobulin (BCAPV). The correlation between BrucellaCapt and BCAPV was 0.982 (p<0.001), with 260 coincident pairs of titers (81%). Areas under the ROC curve for BrucellaCapt and BCAPV with respect to Coombs were 0.969 and 0.960 respectively. BrucellaCapt, BCAPV, Coombs and microagglutination (MAT) tests upon admission were positive in all cases: BrucellaCapt 1/2560, BCAPV 1/2560, Coombs 1/1280, MAT 1/320. The decrease in BrucellaCapt and BCAPV titers over time was pronounced in comparison with Coombs. Cumulative probabilities of persistence 12 months after therapy were: BrucellaCapt 80%, BCAPV 80%, Coombs test 87% and MAT 35%. Serological changes during relapse were detected in 7 cases (88%) by the Coombs test, in 5 cases by BrucellaCapt and BCAPV, and in 3 cases by MAT. BrucellaCapt is a sensitive, specific and simple test for routine use in human brucellosis. Similar results were obtained with BCAPV. However, in some cases of relapse and chronic forms of the disease the slight changes observed in low-affinity antibodies alone are better detected by the Coombs test.
INTRODUCTION

Human brucellosis continues to be a major health problem worldwide. Although this endemic disease is limited to some areas of the Mediterranean basin and developing countries in Asia, Africa and Latin America, sporadic cases may develop in any non-endemic area; thus, the illness is nowadays included among traveling diseases (20).

The definite diagnosis of the disease is based on the isolation of Brucella in blood cultures (11,32) but in certain clinical conditions the microorganism cannot be finally recovered. In the last decade, PCR has emerged as a promising alternative method to confirm the presence of the microorganism (15,23), although its use has not been standardized. Thus, serological tests continue to play a relevant role in the diagnosis and management of patients with brucellosis (11,31). Most classical tests used, along with the ELISA method, offer good detection of anti-lipopolysaccharide (LPS) agglutinating and/or non-agglutinating antibodies. However, problems in serological diagnosis continue, mainly in chronic forms of the disease and in the course of follow-up, when the meaning of persistent titers could be difficult to interpret and no definite criteria of cure have yet been established (2,11,12,17,24,25).

In recent years the new immunocapture agglutination anti-Brucella test (BrucellaCapt) has been reported to detect agglutinating and non-agglutinating antibodies with very high sensitivity (1, 5, 6, 9, 14, 16, 17, 22, 27, 29). It has been suggested as a possible substitute for the anti-human globulin (Coombs) test and, perhaps, as a better marker of disease activity (1, 16, 27, 29). The aim of the present study was to investigate the basis and mechanisms of the high sensitivity of BrucellaCapt (BCAP) and to conduct an accurate evaluation of its behavior in comparison with classical tests in a large group of brucellosis patients with several forms of the disease and across a prolonged follow-up period.
MATERIAL and METHODS

Patients and serum samples

Serum samples from 48 patients diagnosed with brucellosis at Bellvitge Hospital, a tertiary teaching hospital in Barcelona, Spain, were included. These patients were part of a series of cases with brucellosis who were treated and prospectively followed up in our hospital over a period of years and reported elsewhere (2,3). In all patients with positive blood cultures *Brucella melitensis* was identified. The criteria use to select the 48 cases were: 1. availability of frozen serum samples; 2. cases with complicated brucellosis, focal disease or relapse; and 3. prolonged follow-up. The diagnosis of brucellosis was based on clinical findings and positivity of blood cultures for *Brucella* or a microagglutination test (MAT) titer of $\geq 1/160$. Routine blood cultures and serological and clinical evaluations were performed upon admission, at the end of treatment, and at the first, second, third, sixth, ninth, twelfth and eighteenth month after therapy. In cases with clinical relapse an additional sample was obtained for bacteriological and serological studies. Twenty mL blood samples were frozen at -30°C until processing for specific serological studies. Relapse was defined as the reappearance of signs or symptoms of the disease and/or a positive blood culture after therapy. Focal disease was defined as the persistence of signs or symptoms of infection at a particular anatomic site for $>7$ days.

Serological methods

Serological studies were carried out in the microbiology laboratories of the Bellvitge Hospital and the Clinica Universitaria of Navarra. The Rose Bengal test (RB), MAT, Coombs test in microtiter plate, BCAP and a variant of BCAP without microtiter plates coated with anti-total human immunoglobulin (BCAPV) were performed for each sample. All samples from the same patient were processed simultaneously. Patients
whose serum antibody titers upon admission or at 12-18 months after therapy were two or more times higher than the median titers for the whole group of patients at these periods were arbitrarily considered to have high initial or high final titers respectively. For analysis of the probability of persistent significant positivity, titers <1/40 for MAT and titers <1/160 for Coombs, BCAP and BCAPV were considered negative.

RB was performed according to standard procedures (11) and using antigen supplied by the Laboratorio Estatal de Sanidad Animal. Santa Fe (Granada), Spain. MAT was performed on microtiter plates by a double dilution method from an initial 1/20 dilution of the serum sample of phosphate–buffered saline pH 7.2 and using the milk ring test antigen (B. abortus, Central Veterinary Laboratory, Weybridge, Surrey, UK) diluted 1/70 in PBS (7, 8, 11, 13) The plates were incubated for 18-24 hours at 37°C. The Coombs test was performed by microtitration following the method described by Otero et al. (18), with some modifications (14,26,28), and using anti-IgG human immunoglobulins (Operon, Zaragoza) diluted 1:1000 in PBS. BCAP was carried out according to manufacturer’s instructions (Vircell SL, Santa Fe, Granada, Spain). The test is a single-step immunocapture-agglutination assay and consists of microtiter plates coated with anti-human immunoglobulins (IgG and IgA). After addition and dilution of serum in an acid buffer, the antigen suspension included in the Brucellacapt kit (colored B. abortus bacteria killed by formaldehyde treatment) was added and the strips were sealed 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. BCAPV (a variant of BCAP) was performed in an identical way and with the same cell antigen suspension, but using microtiter plates that were not coated with the anti-human immunoglobulin included in the Brucellacapt kit.

Statistical analysis
All data were analyzed by SPSS 12.0 for Windows, except those on Bland-Altman which were analyzed by MedCalc. The Bland-Altman approach was calculated using the method of differences. The data presented were: mean difference, standard vs. test and 95% limits of agreement; and graphical display of difference vs. mean, standard vs. test, not the difference with respect to the standard method. The plot was used to inspect whether the difference and its variance were constant as a function of the average, this being achieved via the correlation of the difference vs. the average; a value near zero implied concordance. Serological data were expressed as median and range of reciprocal titers. Sensitivity, specificity and likelihood ratio (LR) for positive and negative results were calculated. LR values of >10 for positives and <0.1 for negatives were considered conclusive. The kappa statistic was calculated for assessment of agreement between data titers as categorical ratings. Spearman’s correlation coefficient was calculated for the correlation analysis between titers obtained with BCAP, BCAPV, MAT and the Coombs test. These correlations were separately evaluated in samples from the three periods: 1) admission to first month post-therapy; 2) second to sixth month post-therapy; and 3) thereafter. The results for sensitivity and specificity obtained with BCAP and BCAPV titers versus a gold standard titer of Coombs ($\geq 1/160$) and MAT ($\geq 1/80$) for different cut-off points were represented by a curve of diagnostic efficiency (receiver operating characteristic curve or ROC curve). The area under the curve was calculated with a corresponding 95% CI. Survival curves were constructed with the Kaplan-Meier method and were compared by using the log-rank test. A P-value $<0.05$ was considered to be statistically significant.

RESULTS

Characteristics of patients
There were 48 patients, 37 men and 11 women (mean age: 40.83 ± 15.7; range: 15 to 74). Thirty-seven (77%) had positive blood cultures for *Brucella melitensis*. In six patients (shepherds and abattoir workers) the disease was work-related. The time of the disease at diagnosis was 60.3 ± 81.8 days (range: 3 – 365 days). In 20 patients (41.7%) a focal disease was diagnosed: 8 cases of orchitis (21.6% of men), 6 of sacroiliitis (12.5%), 5 of spondylitis (10.4%), 2 of arthritis (4.2%), 2 of prostatitis (5.4%) and 1 of neurobrucellosis (2.1%). There was a relationship between the frequency of focal disease and the time of the disease: 10/36 (27.8%) in patients with a time to 60 days and 10/12 (83.3%) in the group of >60 days (p<.05). Orchitis and sacroiliitis were the most prevalent focal diseases observed in patients with time of evolution ≤15 days.

Three hundred and twenty-one samples were studied serologically (3 to 10 samples per patient; mean ± SD 6.9 ± 1.7 samples). Patients were followed up for a mean time of 18 months (3-36 months); 39 patients had a follow-up ≥12 months.

Eight patients relapsed after treatment (16.6%), with one of them presenting two episodes. Relapse appeared at the first month in four cases, at the third month in two, and there was one episode each in months 6 and 17. Blood cultures were positive for *B. melitensis* at the time of relapse in four of these nine episodes.

Serological titers over time for patients without and with relapse are shown in Figure 1 (A and B respectively); in the last group the titers of the BCAP, BCAPV and Coombs tests decreased more slowly and showed several peaks during this follow-up period; similar results were observed in MAT titers, but to a lesser extent.

**Initial serum antibody titers**

All patients underwent initial serological studies upon admission or during or at the end of treatment. The median serum antibody titers for 35 patients upon admission were:

- BCAP 1/2560 (range, 1/320 – 1/81920),
- BCAPV 1/2560 (range, 1/320 – 1/81920),
- MAT 1/320 (range, 1/320 – 1/81920),
Coombs 1/1280 (range, 1/160 – 1/20480), MAT 1/320 (range, 1/40 – 1/20480). RB, MAT, Coombs, BCAP and BCAPV tests were positive in all cases, but in two patients with positive blood cultures for *B. melitensis* and a disease duration before admission of 90 days, the MAT titer was 1/40.

Eighteen patients had high initial titers of serum antibodies: 9 had MAT titers of ≥1/1280, 14 had Coombs titers of ≥1/5120, 14 had BCAP titers of ≥1/10240 and 12 had BCAPV titers of ≥1/10240.

No correlation was observed between the duration of disease before hospitalization and titers upon admission (p>0.05), although MAT titers did show a tendency to decrease in cases with prolonged disease.

**Relationship between results from classical serological tests and Brucellacapt**

The concordance between the MAT, Coombs, BCAP and BCAPV tests is shown in Figure 2. The Spearman Rho coefficient between BCAP and BCAPV was 0.982 (p<.001), between BCAP and the Coombs and MAT tests it was 0.878 and 0.866, respectively (p<.001), and between BCAPV and the Coombs and MAT tests it was 0.876 and 0.869, respectively (p<.001). The correlation between the Coombs and MAT tests was 0.696 (p<.001). Multiple regression analysis indicated an independent relationship between BCAP or BCAPV (the dependent variables) and the Coombs (p<.005) and MAT (p<.001) tests. No significant differences were observed when these correlations were evaluated separately in samples from different periods of disease evolution.

When coincidence of pairs was evaluated in BCAP and BCAPV, titers were coincident in 260 cases (81%) and non-coincident in 61 (19%) (weighted Kappa 0.926). These non-coincident pairs were: higher BCAP, one dilution in 39 and two dilutions in 3; and
higher BCAPV, one dilution in 19 cases (Table 1). Higher BCAP titers prevailed up to titers of ≥1/640 (p<0.05).

Areas under the ROC curve (AUC) when Coombs was considered as the gold standard ranged between 0.850 and 0.969, the difference between MAT and BCAP or BCAPV being statistically significant (p<0.001); however, no significant difference was observed between BCAP and BCAPV (difference: 0.009; p=0.078). Areas under the ROC curve when MAT was considered as the gold standard were similar in BCAP (0.942) and BCAPV (0.945), but were lower in Coombs (0.853), the difference between BCAP or BCAPV and Coombs being statistically significant (p<0.001) (see Figure 3).

The sensitivity and specificity of BCAP, BCAPV and MAT titers with respect to a gold-standard Coombs titer of ≥1/160 and those of BCAP, BCAPV and Coombs with respect to a gold-standard MAT titer of ≥1/80 are shown in Tables 2 and 3.

**Evolution of serological results over time in 40 patients without relapse**

Antibody titers in serum samples from the 40 patients without relapse decreased over time. In one patient who was in regular contact with sheep a transitory increase of ≥ 2 fold titers was detected in all tests, though he remained symptom-free; an isolated and transitory increased was observed in BCAPV (one sample) and Coombs titers (one sample) in patients who remained well.

The decrease of BCAP and BCAPV titers was very pronounced in comparison with Coombs titers. Thus, if initial BCAP and BCAPV titers were two or more times higher than those of Coombs, by the end of treatment they were already lower. Over the following months these test titers decreased slowly in a similar way, although Coombs titers showed a tendency to persist at higher levels than those of BCAP and BCAPV.

Differences in the evolution of BCAP and Coombs titers were further illustrated when coincidence of pairs was analyzed: before therapy there were 5 coincident pairs, while
in 22 cases BCAP titers were higher and in 7 they were lower than Coombs titers. At the end of therapy or in the first month post-therapy there were 29 coincident pairs, while in 22 cases BCAP titers were higher than Coombs and in 23 they were lower. Over the following months there were 56 coincident pairs, while 30 BCAP titers were higher and 70 lower than Coombs titers.

When this evolution of serological results in patients without relapse was compared between the group of 26 patients without focal disease and the 14 patients with focal disease, a slower decrease in titers in patients with focal disease for all tests was observed; the differences in mean log10 titers upon admission and at 12th month of follow-up were: BCAP, 1.13 vs. 1.59; BACPV, 1.08 vs. 1.53; Coombs, 0.92 vs. 1.17; and MAT, 0.73 vs. 1.32.

The time to negativization was 13 months for MAT, 30 months for BCAP, 29 months for BCAPV and 30 months for the Coombs test. Kaplan-Meier analysis indicated the following cumulative probabilities of persistence of serum antibody titers 12 months after therapy: MAT 35%, BCAP 80%, BCAPV 80% and the Coombs test 87% (Figure 4).

**Seroological outcome in 8 patients with relapse**

Of the 9 episodes among the 8 patients with relapse, serological results were available for eight of them. Serological changes during relapse were detected in 7 cases (88%) by the Coombs test (4 cases with two or more dilutions, 3 cases with one dilution only), in 5 cases by BCAP (2 with two or more dilutions, 3 with one dilution only) and BCAPV (1 with two or more dilutions, 4 with one dilution only) and in 3 cases by MAT (1 with two or more dilutions, 2 with one dilution only) (Table 4). These changes were more evident in bacteremic relapses occurring at least three months after therapy (Figure 5). In patients 1 and 2 (Table 4) clinical reactivation of spondylitis and orchitis was
observed one month after the end of treatment and a concomitant increase in Coombs’
titers from 1/1280 to 1/5120 and from 1/1280 to 1/2560 respectively was detected.

**Persistence of high titers 12-18 months after therapy**

Thirty-three of the 40 patients who did not relapse were able to be followed up
clinically and serologically at least 12-18 months after therapy. Eleven of these patients
had high final serum antibody titers as follows: BCAP ≥1/320 (7 cases), BCAPV
≥1/320 (8 cases), Coombs ≥1/640 (7 cases) and MAT ≥1/80 (5 cases). The remaining 22
patients had low final serum antibody titers. The clinical evaluation was satisfactory
both for patients with high final titers and for those with low final titers. There was a
good correlation between serum antibody titers upon admission and those at 12-18
months; thus, among 15 patients with high initial titers, 9 had high final titers compared
to only two of the 18 patients who did not have high initial titers (P=0.003). The
influence of focal disease in this persistence of high titers was less relevant.
DISCUSSION

The results of our study are in accordance with those reported in previous series regarding the high sensitivity and specificity of BCAP in the diagnosis of human brucellosis (1, 9, 14, 16, 27, 29).

To date, BCAP has been considered an immunocapture-agglutination test whose ability to detect agglutinant and non-agglutinant antibodies successfully has been mostly related to the use of plates coated with anti-human immunoglobulins (1, 9, 14, 16, 27). However, the present study demonstrates that performing a modified but similar test using the same antigen and technique, but with plates that are not coated with immunoglobulins (BCAPV), yields similar results to those of the standard BCAP. Thus, the high ability of BCAP to detect anti-Brucella antibodies is not related specifically to the mechanism of immunocapture, but rather is probably due to the characteristics of the antigen and the acid pH conditions of the test (23, 24). This is a very important finding, not only as regards understanding the mechanisms involved in this test but also because it may be simplified and its cost reduced.

In this study, BCAP had a sensitivity of 100% for the diagnosis of initial disease, given that all patients had titers ≥1/320 at admission (median of 1/2560), which often were several times above the suggested diagnostic threshold of 1/160-1/320.

When the evolution of serological titers was evaluated over time in patients with a good clinical outcome we observed, as reported by others (9, 14, 16, 27), a more pronounced and rapid decrease in BCAP titers in comparison with Coombs, which was already evident in samples at the end of therapy or soon after treatment. The high sensitivity of BCAP, coupled with this rapid decrease in its titers with treatment, suggests that the test mainly detects high affinity antibodies. Furthermore, these findings indicate that BCAP could be a better marker of infection activity and, therefore, a promising substitute for
the Coombs test in the follow-up of patients with brucellosis. However, some additional points should be taken into consideration before such a conclusion is drawn.

After the rapid decrease of BCAP titers detected during the first few months a slower reduction occurs thereafter. Thus, BCAP/BCAPV and Coombs titers remained positive at 12 months post-therapy in 80% and 87% of our patients, respectively, the decrease in titers being particularly slow in patients with focal disease. It should be noted that about 20-25% of patients with a good clinical outcome had BCAP titers of $\geq 1/320$ in samples obtained 12-18 months after therapy, a finding that was mainly related to the detection of very high titers for this test at the initial disease point, as was observed for the Coombs test. Overall, BCAP seems to be more specific than the Coombs test as a marker of disease activity; the detection of a titer $<1/160$ makes present or future activity of the disease very unlikely.

At the time of relapse a serological increase in titers should be observed in order to confirm this diagnosis (30). We previously reported an increase of IgG antibodies in more than 80% of relapsing patients, as detected by IgG ELISA and Coombs tests (2, 21), and it has been suggested that BCAP may have a similar sensitivity (9, 16, 29). However, in the present study, while all patients had BCAP titers $\geq 1/320$ during relapse, serological movement was observed only in 62% of cases with BCAP compared to 87% of cases with Coombs, this change being of $\geq$ two dilutions in 25% and 50% of cases, respectively. The fact that an increase in these titers was detected only in exceptional cases in the group of patients without relapse confers a high specific value on the increase of Coombs titers as markers of relapse. Thus, the sensitivity of Coombs could be higher than that of BCAP, because the Coombs test detects agglutinating and non-agglutinating antibodies, including the ones with high- and low-affinity (4, 10, 19). Taken together, these findings seem to indicate that a movement of low-affinity
antibodies alone may be detected in some patients with active disease; in this regard, we recently reported similar findings in the setting of late hepatosplenic reactivated brucellosis (4, 10).

We conclude that BCAP is a very sensitive, specific and simple test for the routine diagnosis and management of human brucellosis. However, its efficacy is not due to an immune-capture effect, because similar results could be obtained with the same technique but using non-coated anti-immunoglobulin plates; rather, it probably depends on the characteristics of antigen used and the acid pH conditions of the test. This is relevant in terms of cost reduction, because it means that the test can be used with non-coated anti-immunoglobulin plates. As regards detecting mainly high affinity antibodies, BCAP is more specific than the Coombs test. However, if the use of BCAP test as a possible substitute for the Coombs test is considered, it should be taken into account that in some cases of relapse and chronic forms of the disease slight changes of low-affinity antibodies alone are observed, and these are better detected by the Coombs test.
ACKNOWLEDGMENTS

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Table 1. Coincident pairs between BCAP titers and BCAPV titers

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<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>81920</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

% | 0.0 | 7.7 | 13.7 | 13.7 | 18.0 | 14.9 | 9.7 | 5.0 | 9.0 | 5.6 | 12 | 0.9 | 0.3 |
Table 2. Sensitivity, specificity, LR and PV of BCAP, BCAPV and MAT titers with respect to gold standard titers of Coombs of $\geq 1/160$.

<table>
<thead>
<tr>
<th>Serological tests</th>
<th>Titer</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>+ LR</th>
<th>- LR</th>
<th>+ PV</th>
<th>- PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAP</td>
<td>$\geq 160$</td>
<td>91.6</td>
<td>95.9</td>
<td>22.4</td>
<td>0.09</td>
<td>99.2</td>
<td>67.1</td>
</tr>
<tr>
<td>BCAPV</td>
<td>$\geq 160$</td>
<td>91.6</td>
<td>87.8</td>
<td>7.5</td>
<td>0.1</td>
<td>97.7</td>
<td>65.2</td>
</tr>
<tr>
<td>MAT</td>
<td>$\geq 40$</td>
<td>71.9</td>
<td>95.9</td>
<td>17.6</td>
<td>0.29</td>
<td>99</td>
<td>37.9</td>
</tr>
</tbody>
</table>
Table 3. Sensitivity, specificity, LR and PV of BCAP, BCAPV and Coombs titers with respect to gold standard titers of MAT of \( \geq 1/80 \)

<table>
<thead>
<tr>
<th>Serological tests</th>
<th>Titer</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>+ LR</th>
<th>- LR</th>
<th>+ PV</th>
<th>- PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAP</td>
<td>≥ 640</td>
<td>86.7</td>
<td>87.9</td>
<td>7.14</td>
<td>0.15</td>
<td>86.1</td>
<td>88.4</td>
</tr>
<tr>
<td>BCAPV</td>
<td>≥ 640</td>
<td>88.7</td>
<td>89</td>
<td>8.07</td>
<td>0.13</td>
<td>87.5</td>
<td>90.1</td>
</tr>
<tr>
<td>Coombs</td>
<td>≥ 640</td>
<td>84.7</td>
<td>69.4</td>
<td>2.76</td>
<td>0.22</td>
<td>70.6</td>
<td>83.9</td>
</tr>
</tbody>
</table>
Table 4. Antibody titers of BCAP, BCAPV, Coombs and MAT tests in pre-relapse and post-relapse serum samples from 8 cases of relapse

<table>
<thead>
<tr>
<th>Case</th>
<th>Time of relapse (months)</th>
<th>Blood cultures at relapse</th>
<th>Focal disease Initial episode</th>
<th>Focal disease at relapse</th>
<th>BCAP Pre</th>
<th>Post</th>
<th>BCAPV Pre</th>
<th>Post</th>
<th>Coombs Pre</th>
<th>Post</th>
<th>MAT Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1st</td>
<td>Neg</td>
<td>Spondylitis</td>
<td>Spondylitis</td>
<td>2560</td>
<td>2560</td>
<td>2560</td>
<td>2560</td>
<td>1280</td>
<td>5120</td>
<td>320</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>1st</td>
<td>Neg</td>
<td>Orchitis</td>
<td>Orchitis</td>
<td>10240</td>
<td>10240</td>
<td>5120</td>
<td>5120</td>
<td>1280</td>
<td>2560</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>3</td>
<td>1st</td>
<td>Neg</td>
<td>Orchitis</td>
<td>Orchitis</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>1280</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>1st</td>
<td>Neg</td>
<td>Coxitis</td>
<td>Orchitis</td>
<td>320</td>
<td>640</td>
<td>320</td>
<td>640</td>
<td>640</td>
<td>1280</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>3rd</td>
<td>Pos</td>
<td>Sacroiliitis</td>
<td>Orchitis</td>
<td>640</td>
<td>10240</td>
<td>640</td>
<td>10240</td>
<td>320</td>
<td>2560</td>
<td>80</td>
<td>640</td>
</tr>
<tr>
<td>6</td>
<td>3rd</td>
<td>Pos</td>
<td>No</td>
<td>No</td>
<td>1280</td>
<td>2560</td>
<td>1280</td>
<td>2560</td>
<td>1280</td>
<td>5120</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>7</td>
<td>6th</td>
<td>Neg</td>
<td>Spondylitis</td>
<td>Arthritis</td>
<td>5120</td>
<td>2560</td>
<td>5120</td>
<td>2560</td>
<td>10240</td>
<td>5120</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>8</td>
<td>&gt;12th</td>
<td>Pos</td>
<td>Sacroiliitis</td>
<td>No</td>
<td>1280</td>
<td>5120</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>2560</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>Mean</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1280</td>
<td>2560</td>
<td>960</td>
<td>2560</td>
<td>1280</td>
<td>2560</td>
<td>120</td>
<td>160</td>
</tr>
</tbody>
</table>

In bold type: cases with ≥2 increased dilutions; in italics: cases with only one increased dilution
FIG. 1. Serological titers of MAT, BCAP, BCAPV and Coombs tests over time. (A) For patients without relapse and (B) with relapse. The BCAP line is often hidden behind the BCAPV line.
FIG. 2. Bland-Altman graphs showing the level of concordance of BCAP, BCAPV, Coombs and MAT titers. (A) BCAP-BCAPV, (B) BCAP- Coombs, (C) BCAPV- Coombs, (D) BCAP- MAT, (E) BCAPV- MAT, (F) Coombs-MAT.
FIG. 3. (A) Areas under the ROC curve (AUC) of BCAP, BCAPV and MAT when Coombs was considered as the gold standard: BCAP and BCAPV were very similar (0.969 and 0.960), but higher than MAT (0.850) (p<0.001). (B) Areas under the ROC curve (AUC) of BCAP, BCAPV and Coombs when MAT was considered as the gold standard: BCAP and BCAPV were very similar (0.942 and 0.945), but higher than Coombs (0.853) (p<0.001).

A.

B.
FIG. 4. Probability of persistence of positive titers over time for BCAP, BCAPV, Coombs and MAT test. Kaplan-Meier curves of BCAP, BCAPV, Coombs and MAT showing a similar decrease of BCAP and BCAPV titers, both earlier than Coombs; MAT titers decreased even earlier than BCAP and BCAPV.
FIG. 5. (A) Patient with relapse 3 months after the end of therapy (n°6 table 4): note the increase in Coombs titers, while BCAP, BCAPV and MAT titers remained unchanged. (B) Patient with relapse 3 months after the end of therapy (n°5 table 4): note the increase of BCAP, BCAPV and Coombs titers, while MAT titers remained unchanged; note that the increase of BCAP and BCAPV titers was much more pronounced than that of Coombs titers. In both figures the BCAP line is hidden behind the BCAPV line.