

Utility of an immunocapture-agglutination test and an enzyme-linked immunosorbent assay test against cytosolic proteins from *Brucella melitensis* B115 in the diagnosis and follow-up of human acute brucellosis

María Ángeles Mantecón^a, Purificación Gutiérrez^b, María del Pilar Zarzosa^b, Ana I. Dueñas^a, Javier Solera^c, Luis Fernández-Lago^d, Nieves Vizcaíno^d, Ana Almaraz^a, Miguel Angel Bratos^b, Antonio Rodríguez Torres^b, Antonio Orduña-Domingo^{a,b,*}

^aUnidad de Investigación, Hospital Clínico Universitario de Valladolid, 47005 Valladolid, Spain

^bDepartamento Microbiología, Universidad de Valladolid, 47005 Valladolid, Spain

^cMedicina Interna, Complejo Hospitalario de Albacete, 02006 Albacete, Spain

^dDepartamento Microbiología y Genética Molecular, Edificio Departamental, Universidad de Salamanca, 37007 Salamanca, Spain

Received 19 August 2005; accepted 28 November 2005

Abstract

The utility of an immunocapture-agglutination (*Brucellacapt*, Vircell SL, Granada, Spain) test and an enzyme-linked immunosorbent assay IgG, IgA, and IgM (ELISA-IgG, ELISA-IgA, ELISA-IgM) against cytosolic proteins from *Brucella melitensis* B115 (R) was compared with ELISA-IgG, ELISA-IgA, and ELISA-IgM against smooth lipopolysaccharide (S-LPS) from *B. melitensis* 16M (S), serum agglutination test (SAT), and Coombs test in the diagnosis and follow-up for 10 months of 51 patients with acute brucellosis. The sensitivities of ELISA tests against cytosolic proteins varied from 49.0% for ELISA-IgG to 64.7% for ELISA-IgM and were lower than the sensitivities showed by ELISA S-LPS (from 88.2% to 92.2%), SAT (88.2%), Coombs (96.1%), and *Brucellacapt* (98.0%) tests. Specificity was over 93% in all cases. The evolutionary behavior of the SAT, Coombs, and *Brucellacapt* tests was similar. There was a decrease of between 20% and 40% in antibody titer in the 10th month of evolution after treatment. The evolutionary curves of IgG, IgA, and IgM against cytosolic protein increased slightly till the eighth month. The specific IgM and IgA antibodies against protein fractions began to show a drop from the eighth month on, showing levels slightly lower than the initial sera values by the end of the 10th month. In this month, titers of specific IgG against proteins fractions remained higher than the titers showed by the initial sera.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Human Brucellosis; Serology; Cytosolic proteins; Serologic follow-up

1. Introduction

Brucellosis, produced by bacteria of the genus *Brucella*, is a disease that affects both humans and animals. It is one of the most widely spread zoonoses in the world and causes serious problems (Boschiroli et al., 2001).

Clinically, human brucellosis is a systemic disease characterized by great clinical polymorphism and an undulating course, with a strong tendency to present recurrences and evolve to a chronic form (Young, 2005).

Its polymorphism makes clinical diagnosis difficult, requiring laboratory diagnosis to confirm suspicions of the disease.

Etiologic diagnosis of human brucellosis is based on isolating *Brucella* in pathologic products, principally blood (Young, 2005; Yagupsky, 1999). However, the percentage of positive blood cultures depends on the clinical period of the disease; it is low in chronic or evolved phases (Gotuzzo et al., 1986). In addition, *Brucella* bacteria grow slowly and their manipulation carries serious contamination risks for laboratory personnel (Yagupsky et al., 2000). Therefore, in most laboratories, human brucellosis diagnosis is principally based on detection of antibodies against *Brucella*. Among the most widely used serologic techniques are the serum agglutination test (SAT) and the Coombs anti-*Brucella* test, both of which primarily detect antibodies

* Corresponding author. Unidad de Investigación, Hospital Clínico Universitario de Valladolid, Avda Ramón y Cajal 3, 47005 Valladolid, Spain. Tel.: +34-983423063; fax: +34-983423066.

E-mail address: orduna@med.uva.es (A. Orduña-Domingo).

against smooth lipopolysaccharide (S-LPS). However, these techniques present interpretation problems because the antibody titers can remain elevated over long periods after recovery from the disease (Ariza et al., 1992). This causes difficulties in interpreting results in endemic zones, specifically in distinguishing between a current infection and an immune state from a previous infection. It also causes difficulties in diagnosis of recurrences or reinfections (Young, 2005). Similar problems appear in diagnosis of chronic or prolonged evolution forms. *Brucella* also presents cross-reactions with Gram-negative bacteria (Clavijo et al., 2003; Chart and Jenkins, 1999; Cherwonogrodzky et al., 1989) such as *Yersinia enterocolitica* 0:9, *Vibrio cholerae*, *Escherichia coli* O:157, *Pseudomonas maltophilia*, or *Francisella tularensis*. Such reactions can generate false positives in the serologic tests against *Brucella* (Clavijo et al., 2003; Dueñas et al., 2000; Chart and Jenkins, 1999; Colmenero et al., 1994; Díaz and Moriyon, 1989).

Enzyme-linked immunosorbent assay (ELISA) is one method that has been incorporated into serologic diagnosis of brucellosis (Memish et al., 2002; Araj et al., 1988; Saz et al., 1987). Among its advantages are speed and automatization. Individualized studies of each class and subclass of immunoglobulin can be performed as well, so that the contribution of each of them in the distinct phases and evolutionary forms of the disease can be ascertained (Ariza et al., 1992). ELISA also allows detection of antibodies against different bacterial antigenic structures such as corpuscular antigen (Araj et al., 1988; Saz et al., 1987), S-LPS (Baldi et al., 1999; Cloeckert et al., 1999; Fernandez-Lago and Diaz, 1986), or protein antigens (Boschioli et al., 2001; Baldi et al., 1999; Goldbaum et al., 1992; Araj and Kaufmann, 1989). Several authors have reflected how interesting detecting antibodies against protein antigens as an active infection marker can be, given that these antibodies seem to become negative earlier than those against LPS (Boschioli et al., 2001; Baldi et al., 1996a). However, in spite of these characteristics, there have been few studies on brucellosis patients that focus on the behavior of the immune response against *Brucella* protein antigens.

On the other hand, a new technique of immunocapture-agglutination test (*Brucellacapt*, Vircell SL, Granada, Spain) that detects antibodies against whole *Brucella* (mainly against S-LPS) has recently been studied. This technique has shown good sensitivity and specificity in the diagnosis of human brucellosis (Orduña et al., 2000). However, its utility in the follow-up of the disease is unknown.

The aim of this study is to evaluate the diagnostic utility and the evolutionary behavior of an immunocapture-agglutination test (*Brucellacapt*) and an ELISA technique against *Brucella* cytosolic proteins, comparing them with the techniques normally used in brucellosis diagnosis (SAT, Coombs test) and with ELISA tests against S-LPS.

2. Materials and methods

2.1. Clinical samples

The study was performed on 258 sera from 51 patients diagnosed with acute brucellosis. All the patients presented symptoms of brucellosis that was confirmed by laboratory tests. The following criteria were used for including patients in the study as acute brucellosis cases:

- Epidemiologic backgrounds existed for all patients and they presented symptoms compatible with the disease, plus one of the following:
- *Brucella* was isolated from a pathologic patient sample;
- the first serum obtained had a SAT or Coombs anti-*Brucella* test titer $\geq 1/160$; or
- a seroconversion or increase of 4 times the SAT titer was observed.

From the 51 patients, 26 presented positive blood cultures, 4 had seroconversion in the SAT test, and 4 patients showed a 4-fold increase in titer in the SAT.

All the patients evolved favorably in less than 3 months after specific treatment. No patients suffered recurrences in the 12 months after ending treatment.

A blood sample was taken from each patient in the consultation, in which the symptoms corresponding to brucellosis were suspected (initial serum). Blood samples at 2, 4, 6, 8, and 10 months after the start of treatment were also studied (evolutional sera). None of the patients had been diagnosed for brucellosis in the course of the year before initial serum extraction.

To form a negative control group, 412 sera were taken from healthy individuals randomly chosen from areas in which brucellosis is an endemic disease (rural zones in the autonomous region of Castilla y León, Spain). All the serum samples were distributed in aliquots and conserved for less than 3 years at -20°C until use.

2.2. Methods

For each serum, both in the brucellosis patient group and in the negative control group, the presence of antibodies against *Brucella* spp. was determined by slide agglutination test (Linear Chemicals, Barcelona, Spain), Coombs anti-*Brucella* test (Linear Chemicals) (Hall and Manion, 1953), *Brucellacapt* test (Vircell, Granada, Spain) (Orduña et al., 2000), and ELISA-specific IgG, IgM, and IgA against S-LPS of *Brucella melitensis* 16M (S). ELISA studies were also performed to determine the presence of specific antibodies of IgG, IgM, and IgA classes against the cytosolic proteins of the *B. melitensis* B115 (R) strain (Bhonghibat et al., 1970). All the serum samples from each patient were processed simultaneously in each test.

2.2.1. Obtaining S-LPS and *Brucella* protein antigen

S-LPS antigen was obtained from a soy tryptose broth culture of *B. melitensis* 16M (S), according to the method

described by Westphal and Jann (1965) (hot phenol method), modified for *Brucella* (Redfean, 1960). Briefly, phenol-inactivated bacteria (*B. melitensis* 16M) were harvested by centrifugation and washed twice with saline. The bacteria sediment was resuspended in distilled water and mixed with an equal volume of phenol at 66 °C. The mixture was shaken vigorously for 15 min and then centrifuged at $13\,000 \times g$ for 20 min at 4 °C. The LPS contained in the phenol fraction was precipitated with 3 volumes of methanol with 1% methanol saturated with sodium acetate at –20 °C. The precipitant was dialyzed against distilled water. The S-LPS was digested successively with DNase, RNase, and proteinase K, and then obtained by ultracentrifugation at $100\,000 \times g$ for 12 h at 4 °C.

Protein antigen (cytosolic protein) was obtained from a soy tryptose broth culture of *B. melitensis* B115 (R), according to the procedure described by Bhongbhibat et al. (1970). The bacteria were collected by centrifugation, saline-washed, and precipitated with 2 volumes of acetone at –20 °C. The bacteria were harvested by centrifugation, dehydrated by 3 acetone washes, and vacuum dried. The dried bacteria were suspended at 5% (w/v) in 2.5% sodium chloride and shaken in a magnetic stirrer for 3 days at 4 °C. Then, the bacteria were centrifuged at 15 000 rpm for 30 min. The supernatant was precipitated at 4 °C with 3 volumes of ice-cold ethyl alcohol. The precipitate was collected by centrifugation at 15 000 rpm for 30 min, then dialyzed against distilled water and lyophilized. A photograph of the silver-stained polyacrylamide gel electrophoresis (PAGE) of the preparations is presented in Fig. 1.

To test for the absence of *Brucella* LPS in the protein antigen sample, the limulus pyrogenicity test was performed

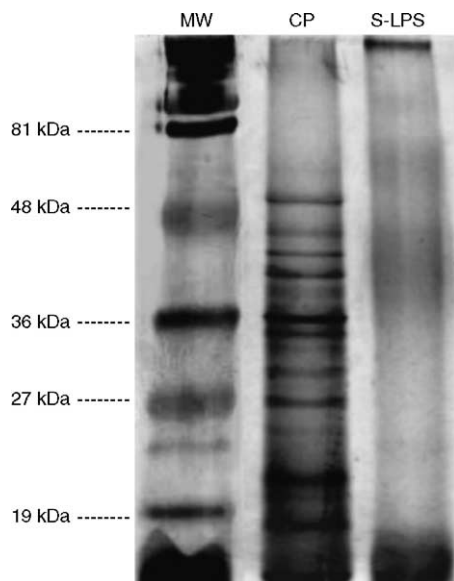


Fig. 1. Silver-stained PAGE gel from smooth-lipopolysaccharide and cytosolic proteins from *B. melitensis* 16 M (LPS) and *B. melitensis* B115 (CP).

(Single Test Limulus Amebocyte Lysate, Pyrogen Plus, Biowhittaker, Walkersville, ME).

2.2.2. SAT and Coombs anti-*Brucella* test

SAT and Coombs tests were performed in test tubes by the 2-fold serial doubling dilutions method, with an initial dilution of 1/20. Commercial corpuscular antigen of *B. abortus* (Linear Chemicals) was used for both tests. SAT reactions were read after 24-h incubation at 37 °C. The highest serum dilution showing more than 50% agglutination was considered the agglutination titer. Coombs tests (Hall and Manion, 1953) were performed on the SAT reactions, after 3 washes with phosphate-buffered saline (PBS; Oxoid, Basingstoke, Hampshire, UK) (pH 7.2) by centrifugation at $3000 \times g$ for 20 min. After the last wash the sediment was resuspended in 1 mL of PBS and then 0.05 mL of previously standardized antihuman total anti-immunoglobulin (Sanofi Pasteur, Marnes-la-Coquette, France) was added to each test tube. The tubes were then mixed and incubated for 24 h at 37 °C. Readings were performed as for the SAT test.

2.2.3. Immunocapture-agglutination (*Brucellacapt*) test

The test was performed according to the manufacturer's instructions. The quantity of 50 µL of each serum dilution was added to the wells of a microplate with a U-shaped base coated with antihuman immunoglobulin. Then 50 µL of the antigen suspension was added (colored *B. melitensis*, killed by formaldehyde treatment). The plates were covered with adhesive sheets and incubated for 24 h at 37 °C in a dark humid chamber. Plate readings were then taken. Positive reactions show agglutination over the bottom of the well. Negative reactions present a pellet on the center of the bottom of the well.

2.2.4. ELISA test against *B. melitensis* B115 (R) protein antigens and *B. melitensis* 16M (S) S-LPS

For the ELISA techniques, 100 µL of antigen solution in PBS at the previously standardized concentration was added to each well of microtiter plates. The plates were incubated for 24 h at 4 °C. Once the antigen had been fixed, a PBS wash cycle was performed and then blocked with a PBS-T/BSA solution (PBS with 0.05% Tween 20 and V fraction bovine serum albumin [BSA; Sigma, St. Louis, MO] at 1%) for an hour at room temperature, and then washed with PBS-T (PBS with 0.05% Tween 20). Next, each well received 100 µL of a serum diluted at 1/100 in PBS-T/BSA. All the problem sera were triple assayed. Two positive control sera from patients with confirmed brucellosis were also placed in each plate; these sera had SAT titers of 1/1280 (high) and 1/160 (low). In addition, each plate received 20 negative control sera from the negative control group, chosen at random from the 412 healthy individuals in that group. All the plates received the same control sera.

Table 1
Diagnostic validity of SAT, Coombs, and *Brucellacapt* tests

		Sensitivity (95% CI)	Specificity (95% CI)	LR (+) ^a (95% CI)	LR (-) ^a (95% CI)
SAT	1/40	88.2 (78.41-98.05)	99.5 (97.96-100)	181.7 (50.17-664.23)	0.11 (0.05-0.23)
	1/80	80.4 (68.51-92.26)	99.5 (97.96-100)	165.6 (45.58-606.63)	0.19 (0.11-0.32)
	1/160	64.7 (50.6-78.8)	99.5 (97.96-100)	133.3 (36.4-491.4)	0.35 (0.23-0.49)
	1/320	47.1 (32.37-61.73)	99.7 (99.16-100)	193.8 (33.94-1114.66)	0.53 (0.39-0.66)
Coombs test	1/40	96.1 (89.77-100)	98.3 (96.93-99.67)	56.5 (27.6-116.61)	0.03 (0.01-0.13)
	1/80	94.1 (86.6-100)	99 (97.96-100)	96.9 (38.01-249.38)	0.05 (0.02-0.16)
	1/160	86.2 (75.84-96.69)	99 (97.96-100)	188.5 (40.5-349.5)	0.13 (0.06-0.25)
	1/320	74.5 (61.56-87.45)	99.5 (98.72-100)	153.5 (42.14-563.4)	0.25 (0.15-0.39)
<i>Brucellacapt</i>	1/40	98.0 (93.25-100)	95.8 (93.83-97.91)	23.7 (15.03-37.87)	0.02 (0.003-0.107)
	1/80	98.0 (93.25-100)	95.8 (93.83-97.91)	23.7 (15.03-37.86)	0.02 (0.003-0.10)
	1/160	92.1 (83.79-100)	98.5 (97.26-99.82)	63.3 (29.21-138.21)	0.07 (0.03-0.18)
	1/320	88.2 (78.41-98.05)	98.7 (97.60-99.96)	72.7 (31.21-170.7)	0.11 (0.05-0.23)

95% CI = 95% confidence interval.

^a LR (+) values above 10 and LR (-) levels under 0.1 are considered clinically useful.

The plates were incubated in a humidity chamber for 2 h at 37 °C. After 5 PBS-T washes, 100 µL of human anti-IgG, IgM, or IgA rabbit antibodies conjugated with horseradish peroxidase (Dako, Cytomation, Cambs, UK) was added. After a new 30-min incubation at 37 °C and further wash cycles, the samples were developed with *o*-phenylenediamine (Sigma). Positivity threshold was established in each plate as the average plus 2 SD of the absorbance of 20 negative control sera group. Absorbance values above this threshold level were considered as positive and those below it as negative.

2.2.5. Evolutional studies

For the SAT, Coombs test, and *Brucellacapt* test evolutionary studies, the percentage of variation of the logarithm of the inverse of the titer of each evolutionary serum in relation to the corresponding initial serum was calculated. A standard curve was prepared with different dilutions of a mixture of sera from patients strongly positive against *Brucella* to determine the antibody levels of each serum against each antigen in the ELISA tests. A value of 1000 U/mL was assigned to this serum mixture. This standard curve was included in all the ELISA plates, and the antibody concentration of the sera in each plate was plotted against the standard curve. Each point of the curves represents the average of the percentages of variation of the evolutionary serum titers with respect to the result from the

initial sera. All the sera from the same patient were triple assayed in the same test.

2.2.6. Statistical analysis

Sensitivity, specificity, and positive and negative likelihood ratios (LRs) were calculated for the results from ELISA techniques and for the SAT, Coombs, and *Brucella capt* tests. Fifty-one initial sera from brucellosis patients were taken as positive control group and 412 sera from healthy individuals living in an area endemic for brucellosis were taken as the negative control group to study the diagnostic validity of the tests.

Sensitivity and specificity were obtained with the version 1.1 Bayesian calculator (<http://www.hsa.es/soft/bayes>). Ninety-five percent confidence intervals were calculated using the Fleiss (1981) method.

Positive and negative LR were calculated with the 1.0 Twobytwo program; 95% confidence intervals were found through the methods of Koopman, (1984), Miettinen and Murnimen (1985), and Gart and Nam (1988). Positive LR [LR (+)] provides information on how many times more probable it is to find a positive result for a patient with a disease than for a healthy individual. The higher the LR (+) level, the more useful the test is to confirm the disease. Negative LR [LR (-)] lets us know how many times more probable finding a negative result in a patient with a disease is than finding it in a healthy individual. The lower

Table 2
Diagnostic validity of ELISA S-LPS and ELISA protein fractions from *Brucella* tests

	Sensitivity (95% CI)	Specificity (95% CI)	LR (+) ^a (95% CI)	LR (-) ^a (95% CI)
ELISA-IgG S-LPS	92.2 (83.79-100)	93.4 (90.93-95.95)	14.06 (9.72-20.43)	0.08 (0.03-0.19)
ELISA-IgM S-LPS	82.3 (70.90-93.79)	93.2 (90.65-95.75)	12.1 (8.29-17.68)	0.18 (0.1-0.32)
ELISA-IgA S-LPS	88.2 (78.41-98.05)	94.2 (91.79-96.5)	15.1 (10.18-22.6)	0.12 (0.06-0.25)
ELISA-IgG-CP	49.0 (34.31-63.72)	97.8 (96.28-99.34)	20.1 (10.38- 39.0)	0.52 (0.38-0.65)
ELISA-IgM-CP	64.7 (50.6-78.8)	97.5 (95.96-99.18)	26.6 (14.13-50.29)	0.36 (0.24-0.50)
ELISA-IgA-CP	54.9 (40.26-69.53)	97.5 (96.28-99.34)	22.6 (11.78-43.27)	0.46 (0.32-0.59)

CP = cytosolic protein.

^a LR (+) values above 10 and LR (-) levels under 0.1 are considered clinically useful.

Table 3

Sensitivity of the serologic tests in brucellosis patients with positive blood culture ($n = 26$)

	Brucellosis patients with positive blood culture and positive serology, n (%)
SAT	22 (84.6)
Coombs test	26 (100)
<i>Brucellacapt</i>	26 (100)
ELISA-IgG S-LPS	23 (88.4)
ELISA-IgM S-LPS	22 (84.6)
ELISA-IgA S-LPS	22 (84.6)
ELISA-IgG-CP	15 (57.6)
ELISA-IgM-CP	16 (61.5)
ELISA-IgA-CP	13 (50)

a LR (–) figure, the more useful the test is in eliminating the disease as a suspect. LR (+) values above 10 and LR (–) levels under 0.1 are considered clinically useful (Jaeschke et al., 1994).

3. Results

3.1. Diagnostic validity

All the initial patient sera were positive (1/20) in the Coombs and *Brucellacapt* tests, whereas 4 sera had negative SAT test results. These 4 patients seroconverted and showed positive results, with titers $\geq 1/160$ in the sera obtained 15 days after initial serum extraction. Within the 412 sera from the negative control group, all the sera resulted negative in the 3 tests, except for 7 sera (1.7%) that were positive in the SAT test with a titer $\geq 1/40$, and 2 of them (0.5%) presented titers of 1/160. Using the Coombs test, 2 sera (0.5%) presented titers of 1/320, and with the *Brucellacapt* test, 5 sera (1.2%) had titers of 1/320. All the sera positive in SAT were also positive in Coombs test and *Brucellacapt*.

Table 1 shows the sensitivity and specificity from each of the techniques, as well as the positive and negative LR. Comparing the SAT, Coombs, and *Brucellacapt* test, the lowest sensitivity was obtained by SAT. At a dilution of 1/40 the SAT test showed a sensitivity of 88.2%, whereas the Coombs and *Brucellacapt* tests presented a sensitivity of

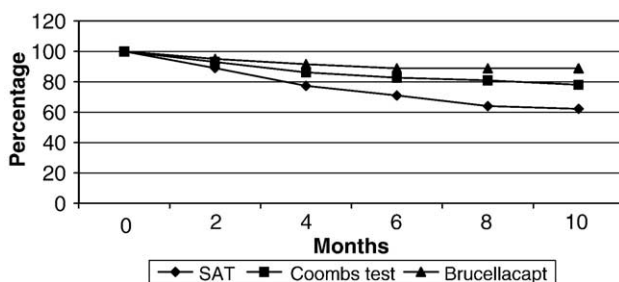


Fig. 2. Evolution of SAT, Coombs test, and *Brucellacapt* in patients with acute brucellosis after the start of treatment. Percentage of variation with respect to the initial serum expressed in percentage of the average of the logarithms of the inverse of the titers.

96.1% and 98.0%, respectively, at the same dilution. Specificity was over 95% in all cases. Likewise, LR (+) was above 23.7 and LR (–) was lower than 0.53 for all cases. However, only Coombs test and *Brucellacapt* showed a LR (–) less than 0.1 and are thus considered clinically useful.

The values for sensitivity, specificity, and LR (+) and LR (–) with ELISA techniques are shown in Table 2. The sensitivities obtained with ELISA tests against S-LPS were similar to those obtained using the Coombs and *Brucella capt* tests, with titers of 1/160 and 1/320 as respective threshold levels for positivity. However, they were higher than those obtained with ELISA against the cytosolic protein antigen of *B. melitensis* B115. Among all the ELISA tests, ELISA-IgG against S-LPS showed the greatest sensitivity (92.2%); ELISA-IgG against the protein fraction obtained a sensitivity of 49.0%. Among the patients with positive blood culture, the most sensitive tests were Coombs and *Brucellacapt* (100% sensitivity in both cases); the least sensitive tests were ELISA-IgG, ELISA-IgA, and ELISA-IgM against cytosolic proteins (57.6%, 61.5%, and 50%, respectively) (Table 3).

Regarding specificity, the ELISA cytosolic protein test result was higher than the 93% obtained by the ELISA-IgG and ELISA-IgM against *B. melitensis* 16M S-LPS. From the 412 negative control sera from healthy individuals, 27 (specificity, 93.2%) were positive with ELISA-IgG against S-LPS and 28 (specificity, 93.4%) were positive in the ELISA-IgM test.

LR (+) was above 9.4 in all cases, whereas LR (–) varied between the 0.08 obtained with ELISA-IgG against S-LPS and the 0.52 reached with ELISA-IgG against the protein fraction of *B. melitensis* B115.

3.2. Antibody evolution

The evolution of the antibody titers determined by SAT, Coombs, and *Brucellacapt* tests was studied, as well as that of the different immunoglobulin classes determined by ELISA against each of the antigens studied.

The evolutionary behavior of the SAT, Coombs, and *Brucellacapt* tests was similar. There was a decrease of

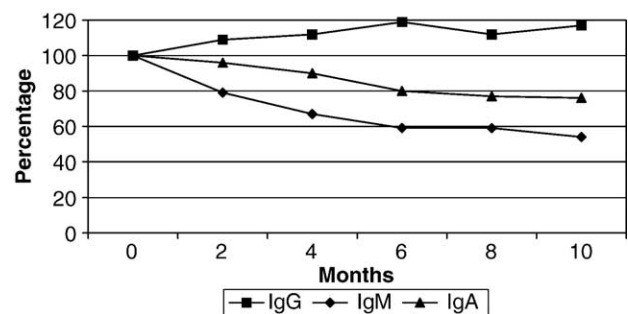


Fig. 3. Evolution of antibodies against S-LPS detected by ELISA. Percentage of variation with respect to the initial serum was expressed as units per milliliter (see Materials and methods).

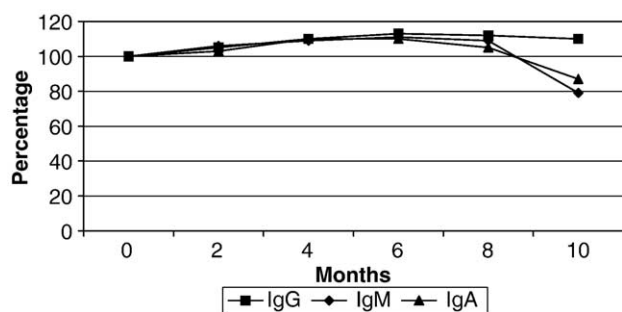


Fig. 4. Evolution of antibodies against cytosolic protein detected by ELISA. Percentage of variation with respect to the initial serum expressed as units per milliliter (see Materials and methods).

between 20% and 40% in antibody titer in the 10th month of evolution (Fig. 2).

The evolutionary profiles of the different immunoglobulin classes against each of the antigens assayed were studied. The IgG against S-LPS presented the highest values throughout the entire evolutionary period, its titers increasing progressively up to 120% above the initial sera (Fig. 3). Titers of IgA against S-LPS traced a descending curve, dropping to values lower than 80% by the end of 10 months, whereas the levels of IgM against S-LPS decrease to 60% of initial values (Fig. 3).

The evolutionary curves of IgG, IgA, and IgM against protein fraction (Fig. 4) increased slightly till the eighth month. The specific IgM and IgA antibodies against protein fraction began to show a drop from the eighth month onward, reaching levels near the 80% of the initial sera values by the end of 10 months after beginning treatment. The IgG antibodies against cytosolic proteins remain higher than 110% (the initial sera values).

4. Discussion

4.1. Diagnostic validity

Serologic diagnosis of human brucellosis involves serious problems. The length of time that antibodies persist after the disease is cured makes distinguishing between past infection and active infection difficult (Ariza et al., 1992). In addition, serologic techniques based on detection of antibodies against *Brucella* S-LPS can give rise to cross-reactions with other bacteria having antigen community at the level of the O-chain polysaccharide S-LPS (Chart and Jenkins, 1999; Díaz and Moriyon, 1989).

We have studied the diagnostic validity of different serologic tests. According to our results, a dilution of 1/40 yields the best sensitivity and specificity in SAT, Coombs, and *Brucellacapt* tests. However, selecting this as a threshold value for positivity can be problematic when interpreting *Brucella* serology in endemic areas. Although specificities reached by these 3 tests were over 95% with the dilution 1/40, in endemic areas the use of the titer 1/40 as a positivity threshold can lead to diagnosing brucellosis in

healthy individuals with low titers against *Brucella* (Kiel and Yousuf Khan, 1987), either from their having recovered from the infection or from having had previous contact with *Brucella* or other antigenically related bacteria (Dueñas et al., 2000; Ariza et al., 1992). In such geographic areas, some authors even recommend using SAT and Coombs titers above 1/320 (Corbel, 1989), although the most frequent suggestions are titers of 1/160 for SAT and 1/320 for Coombs tests (Young, 2005; Corbel, 1989). However, the results from our negative control group showed that 0.2% of the healthy population in an endemic brucellosis zone had SAT titers of 1/320 and 0.7% of them had Coombs titers of 1/320. For lower titers, almost 1% presented Coombs titers $\geq 1/80$, a percentage that reaches 4% in the case of *Brucellacapt*. All the sera positive in SAT were also positive in Coombs and *Brucellacapt* tests.

Using higher titers as a positivity threshold (1/160 and 1/320) decreases test sensitivity, especially with SAT (Orduña et al., 2000). In our study, SAT sensitivity fell to 64.7% when titers of 1/160 were used as a positivity threshold. However, Coombs and *Brucellacapt* test results were not affected as strongly by the use of these positivity thresholds.

SAT titers lower than 1/160 do not always exclude the existence of brucellosis (Young, 1991). Acute brucellosis patients in the earliest phases of the disease can show such titers (Orduña et al., 2000; Cloeckert et al., 1999; Ariza et al., 1992; Young, 1991); even patients with a chronic or prolonged disease evolution can have low SAT titers, although their Coombs and *Brucellacapt* levels are elevated (Orduña et al., 2000).

The fact that there are cross-reactions with other bacteria that *Brucella* presents antigen community with also conditions titer interpretation, although to a lesser extent (Dueñas et al., 2000). This is the case with *F. tularensis* and *Y. enterocolitica* O:9 (Chart and Jenkins, 1999). In our environment, infection by *Y. enterocolitica* O:9 is rare. However, a few epidemic outbreaks of *F. tularensis* have appeared in Spain in the last few years and the appearance of sporadic cases is relatively frequent, but one must bear in mind that a study carried out in Spain on 4825 sera representative of the rural population revealed a prevalence of antibodies against *F. tularensis* of only 0.19% (Gutiérrez et al., 2003).

None of the ELISA tests used in our study improved the sensitivity and specificity shown by the SAT test, except for ELISA-IgG against S-LPS. At any rate, sensitivities obtained by the ELISA tests vary significantly, depending on inherent patient factors, such as time of disease evolution and factors arising from the technical modalities (Ariza et al., 1992; Goldbaum et al., 1992; Araj and Kaufmann, 1989; Araj et al., 1988). For example, Memish et al. (2002) found a sensitivity of 45.6% with a commercial ELISA test on a group of acute and bacteremic patients, whereas Saz et al. (1987) reported a sensitivity of 89% in a group of 208 patients with positive blood culture using complete *Brucella* cells.

In our study, the sensitivity from the ELISA-IgM test against S-LPS (83.3%) is lower than that of the ELISA-IgG test, although this study is based on patients with acute brucellosis. Similar results have been presented in other studies, in which the sensitivity of IgM against S-LPS varies from 79% to 93% even with use of sera from bacteremic patients (Clavijo et al., 2003; Memish et al., 2002; Marrodan et al., 2001; Ariza et al., 1992; Saz et al., 1987). Araj and Kaufmann (1989) and Araj et al. (1988) report sensitivities above 98% with ELISA-IgM tests in patients having acute brucellosis; this percentage drops to 86% with patients in the subacute stage. Apart from whether the clinical classification performed by Araj et al., 1988 can be applied in all cases, it remains clear that 14% of the patients in their study remain undiagnosed if only the ELISA-IgM test is used.

The sensitivity obtained in our study with ELISA-IgA (88%) falls between the 80% obtained by Saz et al. (1987) and the 100% obtained by Ariza et al. (1992) and Araj et al. (1988), the latter with subacute patients.

Positive and negative LRs give us more reliable information about the capability of a technique to diagnose an illness or eliminate it from consideration. According to our data the technique that should be the most useful for confirming the disease is the ELISA-IgA test, as it presents an LR (+) of 15; however, it presents an LR (–) of 0.12 and sensitivity of only 88%. The ELISA-IgG against S-LPS technique offers a better combination of these 2 parameters, with LR (+) of 14.06 and LR (–) of 0.08. These figures, together with the good sensitivity presented by this test, in principle make it the most useful in diagnosis of brucellosis. However, the relatively low specificity of the S-LPS ELISAs compared with the specificity showed by SAT, Coombs test, or *Brucellacapt* argues against its use in the diagnosis of brucellosis in endemic areas. On the other hand, various factors related to the ELISA technique contribute to the marked differences obtained in the studies mentioned. The antigen preparation used, its quality, and the choice of positivity threshold influence sensitivity and specificity (Young, 1991). Although antibodies against S-LPS are detected in all the cases mentioned, in some studies, purified S-LPS (Ariza et al., 1992) has been used, whereas in others, sonicated particle Ag or complete *Brucella* cells have been used (Araj and Kaufmann, 1989; Araj et al., 1988; Saz et al., 1987).

There are also other factors, in addition to technique-related ones, that affect interpretation of results from ELISA S-LPS tests. IgG antibodies against S-LPS can remain elevated for years after the disease is cured (Orduña et al., 2000; Ariza et al., 1992; Goldbaum et al., 1992), making it difficult to distinguish between reinfections or recurrences and residual immune states from previous brucellosis infections. There are no standards that allow a precise quantification of antibody titers either, so no reference value that can be used as a positivity threshold exists. These techniques, which detect antibodies against O-chain poly-

saccharide, are of little use in diagnosing infection by rough *Brucella* species, such as *Brucella canis* (Devi et al., 1987), which has been found to produce cases of human infection (Polt et al., 1982). Some researchers have attempted to solve the problems involved in using diagnostic techniques based on detection of antibodies against S-LPS by switching to tests based on detection of antibodies against *Brucella* protein Ag (Letesson et al., 1997; Baldi et al., 1996b). These protein antigens have been used as allergens in delayed hypersensitivity tests for brucellosis diagnosis (Jones et al., 1973; Bhongbhibat et al., 1970) and more recently in serologic diagnosis of animal brucellosis (Letesson et al., 1997; Debbbarh et al., 1996).

There are few studies on the usefulness of *Brucella* protein antigens for serologic diagnosis of human brucellosis (Cassataro et al., 2002; Goldbaum et al., 1992; Díaz et al., 1976; Díaz, 1974). Using counter-immunoelectrophoresis, Díaz et al. (1976) obtained good reactivity (94%) with sera from patients with brucellosis.

By means of ELISA techniques with a total cytoplasmic fraction, Goldbaum et al. (1992) and Cassataro et al. (2002) also obtained good sensitivities, comparable to those obtained with S-LPS.

Using ELISA techniques against protein antigen of the rough strain *B. melitensis* B115, in this study we observe low sensitivity for brucellosis diagnosis in acute patients. Our figures contrast with those of Goldbaum et al. (1992) (94% for IgG) and Cassataro et al. (2002) (100% for IgG), who used a cytosolic protein antigen. This antigen is obtained by cellular disruption and purification through immunosorbent techniques with monoclonal antibodies against the O polysaccharide of S-LPS. Slightly lower figures (83%) result from using immunosorbently purified *Brucella* lumazine synthase (Cassataro et al., 2002). These results are still much higher than ours, using the protein fraction of the rough strain *B. melitensis* B115 (sensitivity lower than 65%), and higher than the figures they themselves obtain against the recombinant protein rCP24 (Cassataro et al., 2002) and against the protein Omp31 (Cassataro et al., 2004).

In our opinion, the differences found in the sensitivity percentages among the various studies are probably due to the presence of residual S-LPS contaminant. Our study used protein preparation of *B. melitensis* B115 (rough); as is to be expected, no S-LPS was found, even with protein Ag concentrations up to 1 mg/mL. Goldbaum et al. (1992) and Cassataro et al. (2002) obtain their protein antigen through disruption of *Brucella abortus* 19S (smooth strain) and posterior purification by immunosorbent techniques (Goldbaum et al., 1992). These authors detect S-LPS presence by limulus test at a concentration of 0.02 µg/mL in the antigen preparation used to coat the plates. At this concentration we found positive ELISA reactions against S-LPS with sera from brucellosis patients during the standardization of S-LPS plate coating (results not shown). We therefore feel that the high sensitivity found by these authors in using purified cytosolic protein antigen could be

largely due to the presence of contaminant S-LPS. Our results agree more with those obtained using recombinant proteins rCP24 (Cassataro et al., 2002) and Omp31 (Cassataro et al., 2004), which lack contaminant S-LPS.

Neither the cytosolic antigens of our study nor those used in the investigations by other authors mentioned present cross-reactions with Gram-negative bacteria. However, cases of cross-reaction with a bacteria strain have been described (Velasco et al., 1997); the bacterium involved, *Ochrobactrum anthropi*, is phylogenetically related to *Brucella*. Very few cases of infection by this bacterium have been reported and it normally affects individuals with immune deficiency, producing nosocomial infections (Ezzadine et al., 1994). We therefore feel that this fact is not significant.

4.2. Antibody evolution

Analyzing the evolutionary curves of the different tests assayed, we observed that the tests of SAT and ELISA-IgM against S-LPS show a decrease in antibody level, falling to the lowest point at 10 months after the beginning of treatment. In contrast, the tests of Coombs, *Brucellacapt*, and ELISA-IgG against S-LPS and against the protein fractions present, after 10 months' evolution, antibody levels that are similar to or even higher (ELISA-IgG S-LPS and ELISA-IgG against protein fraction) than the initial serum levels. The evolutionary SAT behavior, very similar to that of ELISA-IgM S-LPS test, is because this test detects agglutinant antibodies, mostly of the IgM class against S-LPS from *Brucella*. The presence of IgM antibodies generally indicates a recent infection, as these are the first to appear and also the first to disappear from blood circulation after antigen stimulation ceases. However, in this study, based on patients with acute brucellosis cured within 3 months from beginning treatment, the class IgM antibodies persisted at elevated titers even 10 months after treatment commencement. This means that using specific IgM as the sole test to diagnose human brucellosis could cause false brucellosis diagnoses. This is especially true in endemic areas, where a significant percentage of people may have suffered from an infection by *Brucella*. The fact is that, in our study, 3 (0.7%) of the 412 negative control sera from healthy individuals living in endemic areas presented SAT titers equal to or higher than 1/160, and 28 (6.8%) were positive in the test of ELISA-IgM against S-LPS.

In contrast, the IgG-class antibodies against S-LPS increased, maintaining elevated levels from that point on until the 10th month. Other authors have described this IgG titer stability (Baldi et al., 1996a; Ariza et al., 1992); they find that levels of IgG-class antibodies remain high after clinical recovery. This indicates that the levels of IgG antibodies against S-LPS do not evolve in parallel with disease cure (Baldi et al., 1996a; Ariza et al., 1992). IgG antibodies are therefore not a good serologic marker of active infection, as they can persist for years, even at elevated titers (Ariza et al., 1992).

Protein antigen fractions show hardly any differences in the evolution of each of the specific IgG, IgA, and IgM antibodies. The 3 increased slightly from the fourth month till the eighth month and reach similar levels. The evolution of specific IgA and IgM antibodies after the eighth month marks a gradual, continual decrease in antibody titers; at 10 months after treatment commencement, their levels have fallen to 80–90% of initial serum figures. The titers of specific IgG against protein fraction remain higher than initial sera values. In our experience, it seems that the serologic response against cytosolic proteins starts later than serologic response of *Brucella* superficial antigens like S-LPS.

Acknowledgments

This study was financed by the Agreement for the “Study of brucellosis” from the “Consejería de Sanidad de la Junta de Castilla y León” and the University of Valladolid, and a scholarship from “Red Temática de Investigación Cooperativa sobre Brucelosis” of the “Instituto de Salud Carlos III” (Ref. 03/204).

References

- Araj GF, Kaufmann AF (1989) Determination by enzyme-linked immunosorbent assay of immunoglobulin G (IgG), IgM, and IgA to *Brucella melitensis* major outer membrane proteins and whole-cell heat-killed antigens in sera of patients with brucellosis. *J Clin Microbiol* 27:1909–1912.
- Araj GF, Lulu AR, Khateeb MI, Saadah MA, Shakir RA (1988) ELISA versus routine tests in the diagnosis of patients with systemic and neurobrucellosis. *APMIS* 96:171–176.
- Ariza J, Pellicer T, Pallares R, Foz A, Gudiol F (1992) Specific antibody profile in human brucellosis. *Clin Infect Dis* 14:131–140.
- Baldi PC, Miguel SE, Fossati CA, Wallach JC (1996a) Serological follow-up of human brucellosis by measuring IgG antibodies to lipopolysaccharide and cytoplasmic proteins of *Brucella* species. *Clin Infect Dis* 122:446–455.
- Baldi PC, Giambartolomei GH, Goldbaum FA, Abdón LF, Velikovskiy CA, Kittelberger R, Fossati C (1996b) Humoral immune response against lipopolysaccharide and cytoplasmic proteins of *Brucella abortus* in cattle vaccinated with *B. abortus* S19 or experimentally infected with *Yersinia enterocolitica* serotype O:9. *Clin Diagn Lab Immunol* 3:472–476.
- Baldi PC, Araj GF, Racaro GC, Wallach JC, Fossati CA (1999) Detection of antibodies to *Brucella* cytoplasmic proteins in the cerebrospinal fluid of patients with neurobrucellosis. *Clin Diagn Lab Immunol* 6:756–759.
- Bhonghibat N, Elberg S, Chen TH (1970) Characterization of *Brucella* skin-test antigens. *J Infect Dis* 122:70–82.
- Boschiroli ML, Foulongne V, O'Callaghan D (2001) Brucellosis: A worldwide zoonosis. *Curr Opin Microbiol* 4:58–64.
- Cassataro J, Delpino MV, Velikovskiy CA, Bruno L, Fossati CA, Baldi PC (2002) Diagnostic usefulness of antibodies against ribosome recycling factor from *Brucella melitensis* in human or canine brucellosis. *Clin Diagn Lab Immunol* 9:366–369.
- Cassataro J, Pasquevich K, Bruno L, Wallach JC, Fossati CA, Baldi PC (2004) Antibody reactivity to Omp31 from *Brucella melitensis* in human and animal infections by smooth and rough *Brucellae*. *Clin Diagn Lab Immunol* 11:111–114.
- Chart H, Jenkins C (1999) The serodiagnosis of infections caused by verocytotoxin-producing *Escherichia coli*. *J Appl Microbiol* 86: 731–740.

- Clavijo E, Diaz R, Anguita A, Garcia A, Pinedo A, Smits HL (2003) Comparison of a dipstick assay for detection of *Brucella*-specific immunoglobulin M antibodies with other tests for serodiagnosis of human brucellosis. *Clin Diagn Lab Immunol* 10:612–615.
- Cloekaert A, Tibor A, Zygmunt MS (1999) *Brucella* outer membrane lipoproteins share antigenic determinants with bacteria of the family Rhizobiaceae. *Clin Diagn Lab Immunol* 6:627–629.
- Colmenero JD, Porras J, Cardenas A, Ocon P, Reguera JM, Delgado M, Sedeno J (1994) Evaluation of the chromotitre EIA test in the diagnosis of human brucellosis. *Enferm Infecc Microbiol Clin* 12:60–65.
- Corbel MJ (1989) Microbiology of the genus *Brucella*. In *Brucellosis: Clinical and laboratory aspects*. Eds, EJ Young and MJ Corbel *Brucellosis: Clinical and laboratory aspects*. Boca Raton, Florida: CRC Press, Inc, pp 53–72.
- Cherwonogrodzky JW, Dubray G, Moreno E, Mayer H (1989) Antigens of *Brucella*. In *Animal brucellosis*. Eds, K Nielsen and JR Duncan. Animal brucellosis. New York: CRC Press, pp 19–50.
- Debbbar HS, Cloekaert A, Bézard G, Dubray G, Zygmunt MS (1996) Enzyme-linked immunosorbent assay with partially purified cytosoluble 28-kilodalton protein for serological differentiation between *Brucella melitensis* infected and *B. melitensis* Rev.1-vaccinated sheep. *Clin Diagn Lab Immunol* 3:305–308.
- Devi SJ, Polt SS, Boctor FN, Peter JB (1987) Serological evaluation of brucellosis: Importance of species in antigen preparation. *J Infect Dis* 156:658–661.
- Díaz R (1974) Valor de la prueba de rosa de Bengala y demostración de anticuerpos anti-proteína de *Brucella* en el diagnóstico serológico de brucelosis y yersiniosis. *Med Clin* 63:463–466.
- Díaz R, Moriyón I (1989) Laboratory techniques in the diagnosis of human brucellosis. In *Brucellosis: Clinical and laboratory aspects*. Eds, EJ Young and ML Corbel. *Brucellosis: Clinical and laboratory aspects*. Boca Raton, Florida: CRC Press, Inc, pp 73–83.
- Díaz R, Maravi-Poma E, Rivero A (1976) Comparison of counter-immunoelectrophoresis with other serological tests in the diagnosis of human brucellosis. *Bull World Health Organ* 53:417–424.
- Dueñas AI, Ortega M, Garrote I, de Frutos M, Gutiérrez P, García-Pascual A, et al. (2000) Laboratory diagnosis and serologic course in patients with tularemia. *Med Clin* 114:407–410.
- Ezzadine M, Mourad M, van Ossel C, Logghe C, Squifflet JP, Renault F (1994) An outbreak of *Ochrobactrum anthropi* bacteraemia in five organ transplant patients. *J Hosp Infect* 27:35–42.
- Fernandez-Lago L, Diaz R (1986) Demonstration of antibodies against *Brucella melitensis* 16M lipopolysaccharide and native hapten in human sera by enzyme-linked immunosorbent assay. *J Clin Microbiol* 24:76–80.
- Fleiss JL (1981) Statistical methods for rates and proportions. New York: John Wiley & Sons, Inc.
- Gart JJ, Nam J (1988) Approximate interval estimation of the ratio of binomial parameters: A review and corrections for skewness. *Biometrics* 44:323–338.
- Goldbaum FA, Rubbi CP, Wallach JC (1992) Differentiation between active and inactive human brucellosis by measuring anti protein humoral immune responses. *J Clin Microbiol* 30:604–607.
- Gotuzzo E, Carrillo C, Guerra J, Llosa L (1986) An evaluation diagnostic methods for brucellosis the values of bone marrow culture. *J Infect Dis* 153:122–125.
- Gutiérrez MP, Bratos MA, Garrote JI, Dueñas A, Almaraz A, Alamo R, et al. (2003) Serologic evidence of human infection by *Francisella tularensis* in the population of Castilla y León (Spain) prior to 1997. *FEMS Immunol Med Microbiol* 35:165–169.
- Hall WH, Manion RE (1953) Comparison of the Coombs test with other methods for *Brucella* agglutinins in human serum. *J Clin Invest* 32:96–106.
- Jaeschke R, Guyatt GH, Sackett DL (1994) Users' guides to the medical literature. III. How to use an article about a diagnostic test. A. Are the results of the study valid? *JAMA* 271:389–391.
- Jones LM, Díaz R, Taylor AG (1973) Characterization of allergens prepared from smooth and rough strains of *Brucella melitensis*. *Br J Exp Pathol* 54:492–507.
- Kiel FW, Yousuf Khan M (1987) Analysis of 506 consecutive positive serologic tests for brucellosis in Saudi Arabia. *J Clin Microbiol* 25:1384–1387.
- Koopman PAR (1984) Confidence intervals for the ratio of two binomial proportions. *Biometrics* 40:513–517.
- Letesson JJ, Tibor A, van Eynde G, Wansard V, Weynants V, Denoel P, Saman E (1997) Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. *Clin Diagn Lab Immunol* 4:556–564.
- Marrodan T, Nenova-Poliakova R, Rubio M, Ariza J, Clavijo E, Smits HL, Diaz R (2001) Evaluation of three methods to measure anti-*Brucella* IgM antibodies and interference of IgA in the interpretation of mercaptan-based tests. *J Med Microbiol* 50:663–666.
- Memish ZA, Almuneeff M, Mah MW, Qassem LA, Osoba AO (2002) Comparison of the *Brucella* standard agglutination test with the ELISA IgG and IgM in patients with *Brucella* bacteremia. *Diagn Microbiol Infect Dis* 44:129–132.
- Miettinen O, -Murnimen M (1985) Comparative analysis of two rates. *Stat Med* 4:213–226.
- Orduña A, Almaraz A, Prado A, Gutiérrez MP, García-Pascual A, Dueñas A, Cuervo M, Abad R, Hernández B, Lorenzo B, Bratos MA, Rodríguez Torres A (2000) Evaluation of an immunocapture-agglutination test (*Brucellacapt*) for serodiagnosis of human brucellosis. *J Clin Microbiol* 38:4000–4005.
- Polt SS, Dismukes WE, Flint A, Schaeffer J (1982) Human brucellosis caused by *Brucella canis*: Clinical features and immune response. *Ann Intern Med* 97:717–719.
- Redfearn MS (1960) An immunochemical study of antigens of *Brucella* extracted by the Westphal technique. Madison, USA: University of Wisconsin.
- Saz JV, Beltran M, Diaz A, Agulla A, Merino FJ, Villasante PA, Velasco AC (1987) Enzyme-linked immunosorbent assay for diagnosis of brucellosis. *Eur J Clin Microbiol* 6:71–74.
- Velasco J, Díaz R, Grilló MJ, Barberán M, Marín C, Blasco JM, Moriyón I (1997) Antibody and delayed-type hypersensitivity responses to *Ochrobactrum anthropi* cytosolic and outer membrane antigens in infections by smooth and rough *Brucella* spp. *Clin Diagn Lab Immunol* 4:279–284.
- Westphal O, Jann K (1965) Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. In *Methods in carbohydrate chemistry*. Ed, RL Whistler. Methods in carbohydrate chemistry. New York: Academic Press, pp 83–91.
- Yagupsky P (1999) Detection of Brucellae in blood cultures. *J Clin Microbiol* 37:3437–3442.
- Yagupsky P, Peled N, Riesenberk K, Banai M (2000) Exposure of hospital personnel to *Brucella melitensis* and occurrence of laboratory-acquired disease in an endemic area. *Scand J Infect Dis* 32:31–35.
- Young EJ (1991) Serologic diagnosis of human brucellosis: analysis of 214 cases by agglutination tests and review of the literature. *Rev Infect Dis* 13:359–372.
- Young EJ (2005) *Brucella* species. In *Mandell, Douglas, and Bennett's principles & practice of infectious diseases*. Eds, GL Mandell JE Bennett and R Dolin. Mandell, Douglas, and Bennett's principles & practice of infectious diseases. Philadelphia: Churchill Livingstone, pp 2669–2674.