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Characterization of a Monoclonal Antibody Specific for *Brucella* Smooth Lipopolysaccharide and Development of a Competitive Enzyme-Linked Immunosorbent Assay To Improve the Serological Diagnosis of Brucellosis

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The reactivity of monoclonal antibody (MAb) 12G12 was analyzed in regard to the main biovars of *Brucella* species and some members of the families *Enterobacteriaceae* and *Vibrionaceae* which present serological cross-reactions with the smooth lipopolysaccharide (S-LPS) of *Brucella* species. This MAb was strictly directed against the common specific epitope of the *Brucella* S-LPS. It recognized all of the smooth *Brucella* strains and biovars except *B. suis* biovar 2. In order to improve the specificity of the serological diagnosis of brucellosis, a competitive enzyme-linked immunosorbent assay (cELISA) was developed with the horseradish peroxidase-conjugated MAbs 12G12 and S-LPS of *B. melitensis* Rev1. The specificity of the cELISA was analyzed with 936 serum samples from healthy cattle. The assay was evaluated with sera from heifers ($n = 18$) experimentally infected with *B. abortus* 544. After infection, the performance of the cELISA was in agreement with those of the complement fixation test and the rose Bengal plate test. Finally, the specificity of the assay was also evaluated in regard to false-positive serological reactions by using sera from heifers experimentally infected with *Yersinia enterocolitica* O:9 ($n = 4$) and with field sera presenting false-positive reactions ($n = 74$). The specificity of the cELISA was greater than the specificities of the complement fixation test and the rose Bengal plate test. Indeed, the new assay detected only 31 of the 101 false-positive serum samples detected by at least one serological test.

The serological responses following infection with smooth *Brucella* species are directed predominantly against the smooth lipopolysaccharide (S-LPS). Thus, in humans as well as in animals, the diagnosis of brucellosis is usually based on the detection of specific antibodies against S-LPS (3, 41). This serological detection associated with the slaughter of infected animals was applied with success in the bovine brucellosis eradication program, which is close to its end in most of the European Community countries. However, a problematic aspect of serological detection emerged in the last 5 years in Europe: false-positive serological reactions (FPSRs) either in cattle (4, 7, 20) or in pigs (40). These FPSRs find their origins in the cross-reactivity observed between the S-LPS of *Brucella* species and the S-LPSs of some other bacteria (i.e., *Yersinia enterocolitica* O:9, *Salmonella urbana*, *Vibrio cholerae*, and *Escherichia coli* O:157) (15). Potentially, *Y. enterocolitica* O:9 presents the most serious source of confusion in the diagnosis of brucellosis, because the O chains of the S-LPSs of *Y. enterocolitica* O:9 and *Brucella* species are identical (10, 29). This bacterium was isolated from field animals presenting FPSRs (34, 40) and could be responsible for at least 50% of the FPSRs observed in France (34) and in Belgium (38).

Since this cross-reactivity was described in 1969 (1), several investigators have carried out comparative investigations by different serological methods in order to develop a specific serological diagnosis (16, 24, 25, 31, 32). Until now, none of them has been generally accepted for use in unequivocally distinguishing between the two infections because of limitations with respect to sensitivity, specificity, and/or practicability.

Historically, the smooth-type *Brucella* spp. have been found to contain two distinct antigenic determinants, designated A and M. This characterization had been realized mainly by the agglutination and cross-agglutination patterns obtained with homologous and heterologous *Brucella* antisera and strains (39). By agglutination with monospecific antisera (3) or with monoclonal antibodies (MAbs) specific for the A or the M epitope (21), it was demonstrated that the relative amounts of the two determinants vary among the smooth *Brucella* strains, which are classified into three serotypes, i.e., A^+M^-, A^-M^+, and A^+M^+. Recently, by use of other MAbs, two additional epitopes, common to both S-LPS types, have been described. So, four types of MAbs against S-LPS, those with specificity for the A epitope, the M epitope, and common epitopes cross-reacting (C/Y) or not (C) with S-LPS from *Y. enterocolitica* O:9, have been reported (8, 9, 17, 33). On the basis of these observations, it was possible to develop a competitive enzyme-linked immunosorbent assay (ELISA; cELISA) with a MAb presenting a specificity for the C epitope. Theoretically, this assay could be proposed as an alternative tool for the specific serological diagnosis of brucellosis.

In this report we describe the reactivity of MAb 12G12 (12, 14) with regard to the main species and biovars of the genus *Brucella* and to cross-reactive species. A cELISA was developed with MAb 12G12 and S-LPS from an M epitope-dominant *Brucella* species (*B. melitensis* Rev1), as suggested by Vizcaino et al. (36). The cELISA was compared with the com-

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plement fixation test (CFT) and the rose Bengal plate test (RB), the most widely accepted serological methods, by using 936 serum samples from healthy cattle and heifers experimentally infected with *B. abortus* or *Y. enterocolitica* O:9 and a limited number of false-positive serum samples from animals in the field.

MATERIALS AND METHODS

Bacterial strains. The *Y. enterocolitica* O:9 strain was isolated from a stool specimen by B. Limbourg from the Centre de Dépistage des Maladies Animales, Erpent, Belgium. Others bacterial strains were obtained from the Institut National de Recherches Vétérinaires, Brussels, Belgium (*B. abortus* 544, *B. abortus* W99, *S. urbana*, *E. coli* O:157, and *V. cholerae*), or from the Laboratoire de Pathologie Infectieuse et d'Immunologie, Institut National de la Recherche Agronomique, Nouzilly, France (*B. melitensis* Rev1).

Cattle sera. To evaluate the specificity of the cELISA, 936 serum samples were harvested from animals in a region free of brucellosis and free of FPSRs.

Seventy-four serum samples were taken from cattle presenting FPSRs. These animals were not vaccinated and did not present any symptom of brucellosis, and no abortions were registered on their farms. They were collected by the Centre de Dépistage des Maladies Animales.

Eighteen heifers were infected via the conjunctival route with *B. abortus* 544: 4 heifers pregnant for 5 months were infected with 16.6×10^6 viable cells, 4 heifers (2 nonpregnant heifers and 2 heifers in the first weeks of pregnancy) were infected with 12×10^9 viable cells, and 10 heifers (6 nonpregnant heifers and 4 heifers in the first weeks of pregnancy) were infected with 60×10^6 viable cells. All of the animals were slaughtered on day 80 or day 87 postinoculation, and a bacteriological examination was performed on selected organs and lymph nodes.

Four heifers were infected per os with 10^{12} viable cells of *Y. enterocolitica* O:9. A bacteriological examination of feces was performed every week.

For all experimentally infected animals, serological follow-ups were scheduled at approximately 1-week intervals.

Antigenic preparations. S-LPS fractions of *B. melitensis* Rev1, *B. abortus* W99, *Y. enterocolitica* O:9, *S. urbana*, *E. coli* O:157, and *V. cholerae* were prepared by the phenol-water method (26).

Whole-cell extracts of the *Brucella* strains were prepared by J.-M. Verger and M. Grayon, Laboratoire de Pathologie Infectieuse et d'Immunologie, Institut National de la Recherche Agronomique. Briefly, cell lysis was achieved by boiling in the presence of 1% sodium dodecyl sulfate.

Peroxidase conjugate. MAb 12G12 was produced and purified as described previously (12). It was coupled to horseradish peroxidase (HRP) by a modification of the method of Nakane and Kawaoï as described previously (18).

Serological tests. Classical tests for brucellosis (CFT and RB) were performed as described previously (3, 28). The cutoff values of these tests are 20 IU/ml and any degree of agglutination, respectively.

ELISAs. A direct ELISA with S-LPS was performed as follows. Microtiter plates (2-69620; Nunc, Roskilde, Denmark) were coated by an overnight incubation at 37°C with S-LPS at a final concentration of 1 μg/ml in 100 μl of glycine-buffered saline (GBS; 0.17 M NaCl, 0.1 M glycine, 6 mM NaN_3 [pH 9.2]) diluted 1/5. After five wash cycles with 0.15 M NaCl containing 0.01% Tween 20, the plates were saturated for 3 h at 37°C with 150 μl of phosphate-buffered saline containing 0.1% casein. Fifty microliters of serially diluted HRP-MAb 12G12 in GBS containing 50 mM EDTA and 0.1% Tween 20 (GBS-EDTA-Tween) was added to the plates. The plates were incubated for 60 min at room temperature. The excess reagents were removed by five wash cycles. Citrate-phosphate buffer (0.05 M Na_2HPO_4, 0.025 M citric acid [pH 5]) containing 0.4% *o*-phenylenediamine and 2 mM H_2O_2 was used to visualize the peroxidase activity. The difference in A_{490} and A_{630} was read on a Bio Kinetics Reader EL-340 (Biotek Instruments, Winooski, Vt.).

The cELISA was performed as follows. Plates were coated with S-LPS from *B. melitensis* Rev1 at a final concentration of 0.1 μg/ml. After being washed, the plates were saturated as described above. Twenty-five-microliter serum samples, at 1/5 dilutions in GBS-EDTA-Tween, and 25 μl of GBS-EDTA-Tween containing the appropriate dilution of the HRP-conjugated MAb 12G12 were added to the plates. The plates were incubated for 45 min at room temperature, with a prior agitation for 15 min. The excess reagents were removed, and the peroxidase activity was visualized. Ten dilutions (1/10 to 1/6,120) of a reference serum sample containing 1,000 IU/ml were systematically incorporated into each microtiter plate to define a standard curve. The four-parameter logistic equation, from the Delta Soft software developed by Biometallics (Princeton, N.J.), was used to relate the optical density for each serum sample to this curve and to convert it into competitive units (CU). The cutoff value was calculated with 936 serum samples negative by serological tests (CFT and RB) for brucellosis and was fixed at 50 CU/ml (mean plus 3 standard deviations).

Immunoblot techniques. Five microliters of whole-cell extracts was dotted onto a nitrocellulose membrane. Unoccupied sites were blocked by incubation for 30 min in Tris-buffered saline (TBS; 20 mM Tris hydrochloride, 500 mM NaCl [pH 7.5]) containing 3% bovine serum albumin (BSA) at room temperature. The membranes were incubated for 1 h with HRP-MAb 12G12 in TBS containing 0.05% Tween 20 and 1% BSA. After three washings with TBS containing 0.05% Tween 20 and one washing with TBS, the blots were developed by incubation in a solution of 0.06% (wt/vol) 4-chloro-1-naphthol (Bio-Rad, Richmond, Calif.)–5 mM H_2O_2 in TBS.

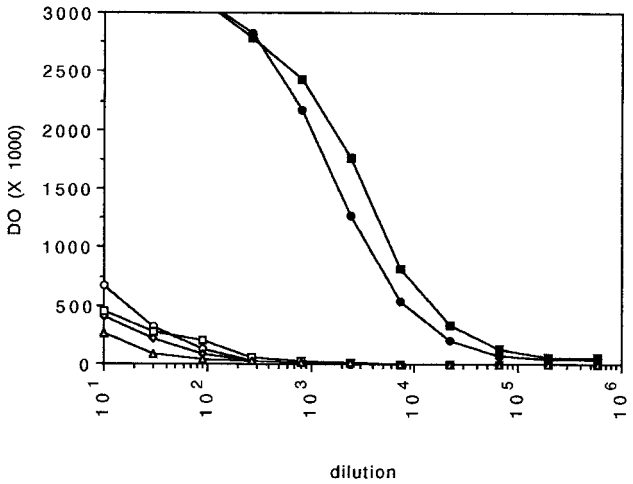


FIG. 1. Binding of HRP-MAb 12G12 to S-LPSs from *B. melitensis* Rev1 (■), *B. abortus* W99 (●), *Y. enterocolitica* O:9 (○), *S. urbana* (◇), *V. cholerae* (□), and *E. coli* O:157 (△). DO, optical density.

Bacteriological analyses. Bacteriological analyses were performed as described previously (37).

RESULTS

Characterization of MAb 12G12. The results obtained by direct ELISA with S-LPS from A epitope- and M epitope-dominant *Brucella* serotypes and from different bacterial species, characterized by their cross-reaction with *Brucella* S-LPS, are shown in Fig. 1. MAb 12G12 recognized epitopes expressed in equal amounts on S-LPS from A epitope- and M epitope-dominant *Brucella* serotypes, *B. abortus* W99 and *B. melitensis* Rev1, respectively. In contrast, MAb 12G12 showed no reactivity with S-LPSs extracted from cross-reactive bacteria (*Y. enterocolitica* O:9, *S. urbana*, *E. coli* O:157, and *V. cholerae*).

The results obtained by studying different *Brucella* strains and biovars by the dot blot technique are provided in Table 1. A positive reaction occurred with all of the naturally occurring smooth *Brucella* strains assayed (*B. melitensis*, *B. abortus*, *B. suis*, and *B. neotomae*) except *B. suis* biovar 2. No significant differences in reaction intensity were found when the different distributions of the A epitope- and M epitope-dominant *Brucella* serovars analyzed were compared. In contrast, rough mutants of *B. melitensis* (H38 R) and *B. abortus* (45/20) and the strains which occur naturally in the rough form (*B. ovis* and *B. canis*) were negative by this assay. Under the same dot blot conditions, *Y. enterocolitica* O:9 was negative (Table 1).

Evaluation of the cELISA using HRP-MAb 12G12 in regard to brucellosis-free animals. In the first step, 936 serum samples obtained from brucellosis-free and FPSR-free farms were used to determine the cutoff value of the cELISA (mean plus 3 standard deviations). On the basis of this value (fixed at 50 CU/ml), one animal was positive by the cELISA, that is, a 99.9% specificity for the cELISA and 100% specificities for CFT and RB.

Evaluation of the cELISA in regard to *Brucella* infections. Four heifers pregnant for 5 months were experimentally infected via the conjunctival route with 16.6×10^6 *B. abortus* 544 organisms (Table 2). The four animals aborted their fetuses 5

TABLE 1. *Brucella* species, biovars, and strains and *Y. enterocolitica* O:9 tested by immunoblotting with HRP-MAb 12G12

Species	Biovar	Strain	S-LPS detected
B. abortus	1	544	+
	1	B19	+
	2	86/8/59	+
	3	Tulya	+
	4	292	+
	5	B 3196	+
	6	870	+
	9	C 68	+
	Rough	45/20	−
B. melitensis	1	16M	+
	1	Rev1	+
	2	63/9	+
	3	Ether	+
	Rough[a]	H38	−
	Rough	B115	−
B. suis	1	1330	+
	2	Thomsen	−
	3	686	+
	4	40	+
	5	513	+
B. canis		RM 6/66	−
B. ovis		BOW 63/290	−
B. neotomae		5 K 33	+
Y. enterocolitica O:9			−

[a] Variant of the smooth H38 strain.

to 9 weeks after the inoculation. They were all positive by the cELISA and RB at 3 weeks postchallenge. The response by CFT was more delayed (all cattle were found to be positive by CFT 4 weeks after the challenge). Eighty-seven days after the inoculation, *B. abortus* was recovered from all the animals which were still positive by the three serological tests.

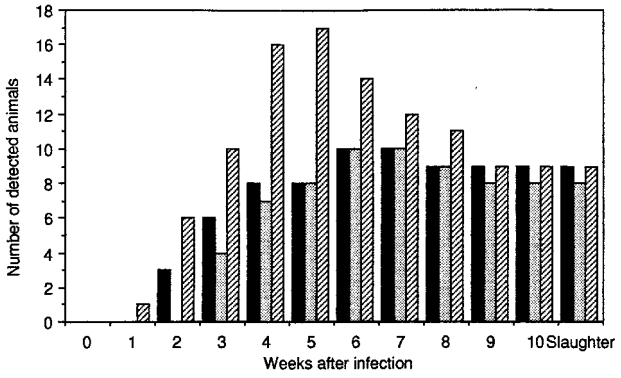
Four other animals (two nonpregnant animals and two animals in the first week of pregnancy) were infected with 12×10^9 *B. abortus* 544 organisms (Table 2). RB was the most efficient test because it detected infection in all of the heifers 14 days after inoculation. By cELISA and CFT, the first positive results occurred on days 14 and 20 postinfection, respectively, but infections in all of the animals were detected only 41 days after inoculation. All except one of the animals remained positive until slaughter (at day 87); for one heifer (animal 8), the serological response observed by CFT was transient. One of the two pregnant heifers (animal 5) aborted their fetuses 32 days after the infection. At the time of slaughter, a specimen from the fetus of the second heifer was culture positive. *B. abortus* was isolated from the lymph nodes of all heifers.

On the basis of the work of Limet et al. (28), 10 heifers were infected via the conjunctival route with 60×10^6 *B. abortus* 544 organisms (Table 2). The conjunctival route and the dose were deliberately chosen to reproduce an infection leading to a weak seroconversion. Only one animal (animal 15) presented a clear and sustained seroconversion by all of the serological tests considered. One heifer (animal 10) did not show any seroconversion. For the remaining animals, the serological response was transient. In fact, both CFT and cELISA detected infection in three animals during only 1 or 2 weeks, while RB seems to have been far more efficient, because it also detected infection in all of the eight remaining animals. Nevertheless, the serological response observed by RB was also transient. For six of the nine heifers which seroconverted, *B. abortus* was isolated from the lymph nodes proximal to the inoculation site, and *B. abortus* was also recovered from the supramammary lymph node (a site distant from the inoculated site) only of the

TABLE 2. Serological and bacteriological results for cattle experimentally infected with *B. abortus* 544

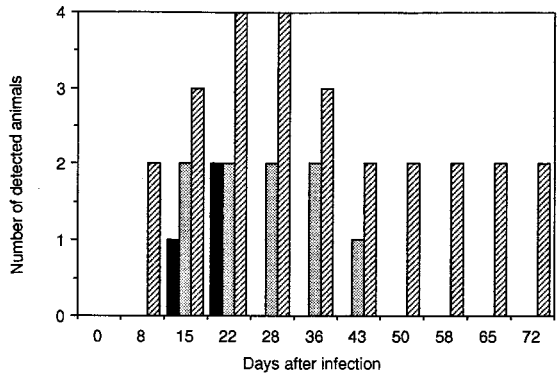
Inoculum and animal no.	State of pregnancy	Day of the first positive bleeding (no. of successive positive bleedings[a])			Bacteriological analysis	
		CFT	RB	cELISA	Cow	Fetus
16.6×10^6 cells						
1	Pregnant for 5 mo	28[b]	7[b]	15[b]	+[c,d]	
2	Pregnant for 5 mo	22[b]	15[b]	22[b]	+[c,d]	
3	Pregnant for 5 mo	28[b]	22[b]	22[b]	+[c]	
4	Pregnant for 5 mo	28[b]	22[b]	15[b]	+[d]	
12×10^9 cells						
5	In the first weeks	20[b]	14[b]	20[b]	+[c,d]	
6	Nonpregnant	20[b]	14[b]	27[b]	+[d]	
7	In the first weeks	41[b]	14[b]	41[b]	+[c,d]	+
8	Nonpregnant	20 (6)	14[b]	14[b]	+[c]	
60×10^6 cells						
9	In the first weeks		35 (1)		−	−
10	Nonpregnant				−	
11	In the first weeks		25 (2)		+[c]	−
12	In the first weeks	35 (1)	25 (3)	42 (1)	+[c]	−
13	Nonpregnant		25 (5)	25 (2)	−	
14	Nonpregnant		20 (3)		+[c]	
15	Nonpregnant	48[b]	25[b]	48[b]	+[c,d]	
16	In the first weeks	42 (2)	20 (5)	42 (2)	−	−
17	Nonpregnant	42 (1)	25 (3)		+[c]	
18	Nonpregnant		25 (5)		+[c]	

[a] Bleedings were done approximately weekly.
[b] Successive positive bleeding until the time of slaughter.
[c] *Brucella* organisms isolated from mandibular and/or parotid lymph nodes.
[d] *Brucella* organisms isolated from supramammary lymph nodes.



	Sensitivities (%)											
cELISA	n.c.	0[a]	16.7	33.3	44.4	44.4	55.5	55.5	50	50	50	64.3[b]
CFT	n.c.	0	0	22.2	38.9	44.4	55.5	55.5	50	44.4	44.4	57.1
RB	n.c.	5.5	33.3	55.5	88.8	94.4	77.8	66.7	61.1	50	50	64.3

FIG. 2. Follow-up of the numbers of animals with infection detected by the different serological tests and evolution of their sensitivities during the experimental infection of 18 heifers with *B. abortus* 544. Symbols: ■, cELISA; ▨, CFT; ▨, RB. *a*, The sensitivity was calculated on the basis of the fact that all the animals were infected. *b*, At the time of slaughter, the sensitivity was estimated by comparison with the bacteriological data. n.c., not calculated.



	Specificities (%)										
cELISA	100	100	75	50	100	100	100	100	100	100	100
CFT	100	100	50	50	50	50	75	100	100	100	100
RB	100	50	25	0	0	25	50	50	50	50	50

FIG. 3. Follow-up of the numbers of animals with infection detected by the different serological tests and evolution of their specificities during the experimental infection of four heifers with *Y. enterocolitica* O:9. Symbols: ■, cELISA; ▨, CFT; ▨, RB.

animal which presented with a positive serological response at the time of slaughter.

Considering that all 18 heifers were infected, the sensitivity of a serological test could be calculated for these 18 animals (Fig. 2). In this case, RB was the most efficient test. Indeed, 5 weeks after the infection, it presented a sensitivity of 94.4% (of the 18 experimentally infected animals, infection was detected in 17 animals), whereas the sensitivities of CFT and cELISA were 55.5%. At 9 weeks after the infection, the sensitivities of the three tests were calculated to be 44.4% for CFT and 50% for RB and cELISA. However, if the serological results obtained for sera collected at slaughter were compared with the bacteriological data (considered as the index of the infection at the end of the experiment), the sensitivities of the different serological tests were 57.1% for CFT (of the 14 heifers from which *Brucella* organisms were isolated at necropsy, infection in 8 animals was detected by CFT) and 64.3% for RB and cELISA (Table 2 and Fig. 2).

Evaluation of the cELISA in regard to FPSRs. Because the cELISA used a MAb specific to the C epitope of the *Brucella* S-LPS, we evaluated the ability of this test to discriminate true brucellosis from FPSRs.

Four heifers were experimentally infected with *Y. enterocolitica* O:9 (Fig. 3). RB detected infection in two animals at day 8, and all of the animals presented a FPSR 22 days after the infection and for at least 2 weeks. Two of these animals were still positive 72 days after the trial. Two heifers had developed a significant response to CFT at day 15, and these responses lasted for 3 and 4 weeks, respectively. The cELISA detected infection in the same two animals, but the serology was still more transient (one or two positive successive bleedings). *Y. enterocolitica* O:9 was isolated from the feces of all animals infected with this organism for 2 or 3 weeks after the trial.

Seventy-four serum samples from animals in the field which presented FPSRs by RB and/or CFT were chosen to study the specificity of the cELISA (Table 3). A total of 93.2 and 58.1% of these serum samples presented positive results by RB and CFT, respectively, whereas cELISA detected only 36.5% of the false-positive serum samples.

DISCUSSION

A possible strategy for circumventing the cross-reactivity caused by anti-S-LPS antibodies is to base the diagnosis on selected *Brucella* proteins. Such antigens, identified by either Western blotting (immunoblotting), ELISA (13, 27, 35, 42), or screening an expression library (23), were evaluated as diagnostic tools with limited success. In fact, it appears that, compared with the anti-S-LPS response, the antiprotein antibody response is too heterogeneous and too delayed to be of great help in the serological diagnosis of brucellosis. Consequently, we chose to build a specific serological test based on the S-LPS epitope which is strictly specific for *Brucella* species.

The MAb 12G12 used in the present work is directed against this specific epitope. First, it recognized all of the smooth *Brucella* biovars except *B. suis* biovar 2. Another MAb which presented the same reactivity as MAb 12G12 toward the main *Brucella* strains and biovars was also described (MAb BM/40) (22). Compared with *B. suis* biovars 1, 3, and 4, which are extremely pathogenic for humans, *B. suis* biovar 2 is a relatively poor pathogen for cattle and humans (2). Thus, the failure of MAb 12G12 to recognize this biovar is probably not a problem. Second, MAb 12G12 was strictly specific for the C epitope because it did not react with either the S-LPS of *Y. enteroco-*

TABLE 3. Serological reactivities of 74 serum samples that reacted nonspecifically by RB and/or CFT[a]

Test	No. of false-positive serum samples	Specificity (%)
RB	69	6.8
CFT	43	41.9
RB + CFT	74	NC[b]
cELISA	27	63.5

[a] All sera were tested by RB, CFT and cELISA.
[b] NC, not calculated.

litica O:9 or the S-LPSs of other bacteriological species sharing S-LPS determinants with *Brucella* species. So, a cELISA with MAb 12G12 could be an alternative tool to avoid FPSRs, while it would still be able to detect infections with A^+M^-, A^-M^+, or A^+M^+ *Brucella* species.

The sensitivity of the cELISA was analyzed with sera from experimentally infected cattle. Only the conjunctival route of infection was used. As expected, the dose of viable *B. abortus* used for the infections as well as the state of pregnancy of the heifers had an influence on the kinetics and the intensity of the serological response. Under all of these different conditions, which experimentally reproduced different situations in the field, RB as well as the indirect ELISA (data not shown) detected infected animals earlier than the other serological tests did. The cELISA seems to detect infected animals earlier than CFT does. However, when a low dose was used to infect nonpregnant heifers or heifers in the first weeks of pregnancy, all of the serological tests showed a low sensitivity with regard to bacteriological analysis. These experimental infections had probably reproduced the phenomenon of latency.

In order to evaluate the potential of the cELISA as a new assay for solving the problem of FPSRs, the specificity of the cELISA was evaluated with sera from cattle experimentally infected with *Y. enterocolitica* O:9 and with sera from animals in the field presenting false-positive results. RB, which detected animals with brucellosis earlier than the other serological tests, was also the test by which more false-positive results were obtained. Indeed, when all of the sera which presented false-positive results by at least one serological test were considered ($n = 101$), the cELISA with MAb 12G12 reduced drastically the problem of FPSRs because the specificity of this test was 69.3%, compared with specificities of 5.9, 24.8, and 48.5% for RB, the indirect ELISA (data not shown), and CFT, respectively.

A possible explanation of the lack of specificity of the cELISA in the case of FPSRs could be the fact that some antibodies induced by a *Y. enterocolitica* O:9 infection were able to interfere by steric hindrance with the specific binding of MAb 12G12. Indeed, the O chains of *Y. enterocolitica* O:9 and *Brucella* spp. consist of unbranched polysaccharides of 4,6-dideoxy-4-formamido-D-mannopyranosyl residues (10). The single difference between these two chains are the links between the residues (α-1,2 links for the O chain of *Y. enterocolitica* O:9 and α-1,2 and α-1,3 links in the case of the O chains of *Brucella* spp.) (30). The sequence of two to four residues with α-1,2 links forms the C/Y epitope (8) which is the only one common to the S-LPS of *Y. enterocolitica* O:9 and to the *Brucella* S-LPS used in the present cELISA. Thus, the antibodies produced by an infection with *Y. enterocolitica* O:9 and able to inhibit the binding of MAb 12G12 could be directed against the C/Y epitope. Thus, the capacities of MAbs specific to the C/Y epitope of *Brucella* S-LPS to inhibit the binding of MAb 12G12 will be studied to understand the nonspecific competitions.

In conclusion, the performance of the cELISA was in agreement with those of other serological tests because the cELISA detected the same infected animals. The cELISA is a versatile approach unrestricted to the animal species analyzed. It offers the advantage of being a simple, rapid assay. Although it was proved to be far more specific than other available serological tests, some cases of FPSRs were still refractory to the procedure. Until now, specific diagnoses were realized only with cellular tests (i.e., delayed-type hypersensitivity test, lymphocyte transformation tests, or gamma interferon assay [5, 11, 37]), which detect infected animals as early as serological tests do (37). However, because neither the cellular tests nor the serological tests were able to detect all of the infected animals at every stage of infection, these two approaches remain complementary (6, 19, 37). Therefore, the improvements to the specificity of serological diagnosis are still necessary.

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REFERENCES

1. **Ahvonen, P., E. Jansson, and K. Aho.** 1969. Marked cross-agglutination between *Brucellae* and a subtype of *Yersinia enterocolitica*. Acta Pathol. Microbiol. Scand. **75:**291–295.
2. **Alton, G. G.** 1990. Brucellosis suis, p. 411–422. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
3. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris.
4. **Benet, J. J., C. Massard, and B. Garin-Bastuji.** 1991. Réactions sérologiques atypiques dans le dépistage de la brucellose bovine. Epidemiol. Sante Anim. **19:**97–130.
5. **Bercovich, Z., W. Lagendijk, and B. A. Bokhout.** 1989. Evaluation of a delayed hypersensitivity test for the diagnosis of *Brucella* infection in cattle. Vet. Immunol. Immunopathol. **21:**213–218.
6. **Bercovich, Z., and E. A. Ter Laak.** 1990. An evaluation of the delayed-type hypersensitivity test for diagnosing brucellosis individual cattle: a field study. Vet. Microbiol. **22:**241–248.
7. **Bertin, Y.** 1993. Sérologies atypiques et prophylaxie de la brucellose bovine dans le département du Puy-de-Dôme. Med. Mal. Infect. **23:**513–515.
8. **Bundle, D. R., J. W. Cherwonogrodzky, M. A. J. Gidney, P. J. Meikle, B. P. Malcom, and T. Peters.** 1989. Definition of *Brucella* A and M epitopes by monoclonal typing reagents and synthetic oligosaccharides. Infect. Immun. **57:**2829–2836.
9. **Bundle, D. R., M. A. J. Gidney, M. B. Perry, J. R. Duncan, and J. W. Cherwonogrodzky.** 1984. Serological confirmation of *Brucella abortus* and *Yersinia enterocolitica* O:9 O antigens by monoclonal antibodies. Infect. Immun. **46:**389–393.
10. **Caroff, M., D. R. Bundle, and M. B. Perry.** 1984. Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. Eur. J. Biochem. **139:**195–200.
11. **Chukwu, C. C.** 1985. Differentiation of *Brucella* and *Yersinia enterocolitica* serotype O:9 infections: use of lymphocyte transformation test. Int. J. Zoonoses **12:**126–135.
12. **Cloeckaert, A., P. De Wergifosse, G. Dubray, and J. N. Limet.** 1990. Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labelling for electron microscopy and enzyme-linked immunosorbent assay. Infect. Immun. **60:**312–315.
13. **Cloeckaert, A., P. Kerkhofs, and J. N. Limet.** 1992. Antibody response to *Brucella* outer membrane proteins in bovine brucellosis: immunoblot analysis and competitive enzyme-linked immunosorbent assay using monoclonal antibodies. J. Clin. Microbiol. **30:**3168–3174.
14. **Cloeckaert, A., M. S. Zygmunt, G. Dubray, and J. N. Limet.** 1993. Characterization of O-polysaccharide specific monoclonal antibodies derived from mice infected with the rough *Brucella melitensis* strain B115. J. Gen. Microbiol. **139:**1551–1556.
15. **Corbel, M. J.** 1985. Recent advance in the study of *Brucella* antigens and their serological cross-reactivity. Vet. Bull. **55:**927–942.
16. **Corbel, M. J., and G. A. Cullen.** 1970. Differentiation of the serological response to *Yersinia enterocolitica* and *Brucella abortus* in cattle. J. Hyg. Camb. **68:**519–530.
17. **Douglas, J. T., and D. A. Palmer.** 1988. Use of monoclonal antibodies to identify the distribution of A and M epitopes on smooth *Brucella* species. J. Clin. Microbiol. **26:**1353–1356.
18. **Dubray, G., and J. Limet.** 1987. Evidence of heterogeneity of lipopolysaccharides among *Brucella* biovars in relation to A and M specificities. Ann. Inst. Pasteur/Microbiol. **138:**27–37.
19. **Fensterbank, R.** 1977. Diagnostic allergique de la brucellose bovine. 2. Utilisation du test allergique dans les troupeaux infectés. Ann. Rech. Vet. **8:**195–201.
20. **Garin-Bastuji, B.** 1993. Le dépistage de la brucellose des ruminants et ses difficultés. Point Vet. **25:**115–124.
21. **Garin-Bastuji, B., R. A. Bowden, G. Dubray, and J. N. Limet.** 1990. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

analysis of smooth-lipopolysaccharide heterogeneity among *Brucella* biovars related to A and M specificities. J. Clin. Microbiol. **28:**2169–2174.
22. **Greiser-Wilke, I., and V. Moennig.** 1987. Monoclonal antibodies and characterization of epitopes of smooth *Brucella* lipopolysaccharides. Ann. Inst. Pasteur/Microbiol. **138:**549–560.
23. **Hemmen, F., V. Weynants, T. Scarcez, J.-J. Letesson, and E. Saman.** 1995. Cloning and sequence analysis of a newly identified *Brucella abortus* gene and serological evaluation of the 17-kilodalton antigen that it encodes. Clin. Diagn. Lab. Immunol. **2:**263–267.
24. **Hurvell, B.** 1973. Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. Biological and chemical investigations of lipopolysaccharides from *Brucella abortus* and *Yersinia enterocolitica* type IX. Acta Pathol. Microbiol. Scand. **81:**105–112.
25. **Hurvell, B., and A. A. Lindberg.** 1973. Immunochemical studies on the cross-reactions between *Brucella* species and *Yersinia enterocolitica* type IX. Contrib. Microbiol. Immunol. **2:**159–168.
26. **Leong, D., R. Diaz, K. Milner, J. Rudbach, and J. B. Wilson.** 1970. Some structural and biological properties of *Brucella* endotoxin. Infect. Immun. **1:**174–182.
27. **Limet, J. N., A. Cloeckaert, G. Bezard, J. Van Broeck, and G. Dubray.** 1993. Antibody response to the 89-kDa outer membrane protein of *Brucella* in bovine brucellosis. J. Med. Microbiol. **39:**403–407.
28. **Limet, J. N., P. Kerkhofs, R. Wijffels, and P. Dekeyser.** 1988. Le diagnostic sérologique de la brucellose bovine par ELISA. Ann. Med. Vet. **132:**565–575.
29. **MacMillan, A.** 1990. Conventional serological tests, p. 153–197. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
30. **Meikle, P. J., M. B. Perry, J. W. Cherwonogrodzky, and D. R. Bundle.** 1989. Fine structure of A and M antigens from *Brucella* biovars. Infect. Immun. **57:**2820–2828.
31. **Mittal, K. R., I. D. Ricciardi, and I. R. Tizard.** 1980. Indirect hemagglutination employing enterobacterial common antigen and *Yersinia* somatic antigen: a technique to differentiate brucellosis from infections involving cross-reacting *Yersinia enterocolitica*. J. Clin. Microbiol. **11:**149–152.
32. **Mittal, K. R., and I. R. Tizard.** 1980. Studies on the relationship between *Yersinia enterocolitica* IX and *Brucella abortus* agglutinins in naturally infected animals. Res. Vet. Sci. **28:**311–314.
33. **Palmer, D. A., and J. T. Douglas.** 1989. Analysis of *Brucella* lipopolysaccharide with specific and cross-reacting monoclonal antibodies. J. Clin. Microbiol. **27:**2331–2337.
34. **Reynauld, A., C. Delmas, and J. M. Vidon.** 1993. Isolement fécal de *Yersinia enterocolitica* O:9 chez plus de 50% des ruminants présentant une sérologie atypique vis-à-vis de *Brucella abortus*. Med. Mal. Infect. **23:**516–519.
35. **Tabatabai, L. B., and S. G. Hennager.** 1994. Cattle serologically positive for *Brucella abortus* have antibodies to *B. abortus* Cu-Zn superoxide dismutase. Clin. Diagn. Lab. Immunol. **1:**506–510.
36. **Vizcaino, N., A. Chordi, and L. Fernandez-Lago.** 1991. Characterization of smooth *Brucella* lipopolysaccharides and polysaccharides by monoclonal antibodies. Res. Microbiol. **142:**971–978.
37. **Weynants, V., J. Godfroid, B. Limbourg, C. Saegerman, and J.-J. Letesson.** 1995. Specific bovine brucellosis diagnosis based on an in vitro antigen-specific gamma interferon production. J. Clin. Microbiol. **33:**706–712.
38. **Weynants, V., A. Tibor, P. A. Denoel, C. Saegerman, J. Godfroid, and J.-J. Letesson.** 1996. Infection of cattle with *Yersinia enterocolitica* O:9, a cause of the false positive serological reactions in bovine brucellosis diagnosis tests. Vet. Microbiol. **48:**707–772.
39. **Wilson, G. S., and A. A. Miles.** 1932. The serological differentiation of smooth strains of the Brucella group. Br. J. Exp. Pathol. **13:**1–13.
40. **Wrathall, A. E., E. S. Broughton, K. P. W. Gill, and G. P. Goldsmith.** 1993. Serological reactions to *Brucella* species in British pigs. Vet. Rec. **132:**449–454.
41. **Wright, P. F., and K. H. Nielsen.** 1990. Current and future serological methods, p. 305–320. *In* L. G. Adams (ed.), Advances in brucellosis research. Texas A&M University Press, College Station.
42. **Zygmunt, M., F. B. Gilbert, and G. Dubray.** 1992. Purification, characterization, and seroactivity of a 20-kilodalton *Brucella* protein antigen. J. Clin. Microbiol. **30:**2662–2667.