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Evaluation of Allergic and Serological Tests for Diagnosing *Brucella melitensis* Infection in Sheep

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A total of 291 unvaccinated sheep from *Brucella melitensis*-infected flocks were examined for delayed-type hypersensitivity (DTH) responses with Brucellergene commercial allergen and with cold saline extract and cytosol from rough *B. melitensis* 115, and their sera were tested in the rose bengal test (RBT), complement fixation test (CFT), and enzyme-linked immunosorbent assay (ELISA) with lipopolysaccharide. DTH reactions were maximal after 72 h, with no intensity differences among allergens, inoculation sites (eyelid and tail), and doses tested. There were no differences in the results recorded by visual inspection and palpation of inoculation sites, by measuring skin thickness with a caliper, or by microscopic examination of samples taken at necropsy. Six days after DTH testing, anergy was observed in 100% of the animals, and 100% reactivity was recovered only after 24 days. All animals were necropsied, and thorough bacteriological searches were performed. The sensitivities found with the 140 animals from which *B. melitensis* was isolated were ELISA, 100%; DTH, 97.1%; RBT, 92.1%; and CFT, 88.6%. Those results put into question the value of RBT and CFT as screening and confirmatory tests for sheep brucellosis and at least indicate that their standardization should be modified. For 151 tested sheep from which *B. melitensis* was not isolated, the percentages of positive animals were ELISA, 100%; DTH, 94.0%; RBT, 57.6%; and CFT, 53.6%. All tests were negative for 100 tested sheep from *Brucella*-free flocks. The different results of bacteriological and immunological tests suggest the usefulness of developing indirect tests able to distinguish truly infected animals from those that have developed an immunological response.

Brucella melitensis causes an important infectious disease affecting mostly small ruminants and humans. The diagnosis of the disease is usually carried out by serological testing, and it is assumed that the serological tests used for the diagnosis of *Brucella abortus* infection in cattle are also adequate for the diagnosis of *B. melitensis* infection in the small ruminants. Accordingly, the rose bengal test (RBT) and the complement fixation test (CFT) are widely used for the serological diagnosis of sheep brucellosis (12, 25), and they are the official tests in European Community countries. However, there are contradictory reports on the value of the above tests when applied to sheep (2, 5, 16, 18, 27, 29, 30), possibly because of the paucity of data obtained with animals shown to be actually infected by bacteriological isolation of *B. melitensis*. In addition to serological tests, delayed-type hypersensitivity (DTH) tests have been applied to the diagnosis of *B. melitensis* infection in sheep (11, 15, 20, 21, 23) but the diagnostic value of DTH tests has seldom been assessed in bacteriologically studied animals (11). Therefore, the aim of this work was to compare several serological and DTH tests for the diagnosis of *B. melitensis* infection in sheep, and to evaluate the results with respect to a bacteriological study carried out at necropsy.

MATERIALS AND METHODS

Allergens. (i) Cold saline allergen (CSA). CSA was obtained by the modification of the method of Bhongbhibhat et al. (4) described by Jones et al. (20). The rough strain *B. melitensis* 115 was grown in tryptic soy broth, and the cells were harvested by tangential flow filtration (Pellicon unit, PTHK000C5 filter, Millipore Corp., Bedford, Mass.) and dried with acetone. The dried cells were resuspended (5%, wt/vol) in 2.5% NaCl and held at 4°C for 48 h with continuous stirring. After centrifugation (13,000 × g, 30 min, 4°C), the supernatant was mixed with 3 volumes of cold ethanol and the mixture was held at 4°C for 24 h. The precipitate formed was sedimented (5,000 × g, 4°C, 15 min), suspended in distilled water, and dialyzed against 3 × 100 volumes of deionized distilled water. The solution was then ultracentrifuged (100,000 × g, 6 h), and the supernatant (CSA) was freeze-dried.

(ii) Cytosol. *B. melitensis* 115 cells grown as described above were harvested in the logarithmic phase of growth and washed once with cold saline. A thick cell suspension prepared in 10 mM phosphate-buffered saline (PBS), pH 7.2, was supplemented with DNase (type II; Sigma Chemical Co., St. Louis, Mo.) and RNase (Miles Laboratories, Inc., Elkhart, Ind.) (50 μg/ml each), and the cells were disintegrated in a 40K French pressure cell (SLM Instruments, Inc., Urbana, Ill.) operating at 32,200 psi (4,600 kg/cm^2) of internal pressure. After incubation for 4 h at 37°C with magnetic stirring, the cell envelopes were sedimented (80,000 × g, 2 h, 4°C), and the supernatant was held at 4°C for 24 h before being ultracentrifuged again under the same conditions. The new supernatant (cytosol) was dialyzed against 10 mM sodium phosphate buffer, pH 7.2, and freeze-dried.

(iii) Brucellergene. Brucellergene OCB (batch 26 N 151) was kindly provided by Rhone-Merieux (Lyon, France). According to the manufacturer, the allergen is prepared from *B. melitensis* 115 and the product contains 2,000 U of protein per

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ml and 0.1 mg of sodium merthiolate per ml in 0.15 M NaCl. For protein and lipopolysaccharide (LPS) analysis (see the details below), Brucellergene was precipitated with 5 volumes of acetone in cold conditions, and the precipitate was freeze-dried.

Analytical methods. Total protein was determined by the method of Lowry et al. (24), with bovine serum albumin used as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue or Coomassie blue-silver stain for proteins were performed as described elsewhere (7, 22). Bovine serum albumin (69K; apparent molecular weight, 69,000), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21K), and lysozyme (14.4K) were used as molecular weight standards. The presence of rough LPS was tested by SDS-PAGE followed by periodate-silver staining (31); the sensitivity of the method was 80 ng for *B. abortus* 45/20 rough LPS obtained by the method described by Galanos et al. (17), equivalent to 0.2% rough LPS in the sample.

Serological tests. The RBT was performed on glossy white tiles with the standard *B. abortus* antigen (3) but with a mixing of 75 μl of sera and 25 μl of antigen, a modification that increases sensitivity (5). The CFT was performed on microplates with a standard suspension of smooth *B. abortus* cells as antigen and by the warm procedure described by Alton et al. (3). Serum dilutions of 1/4 or higher showing 50% or less hemolysis (20 CEC units) were considered positive.

The indirect enzyme-linked immunosorbent assay (ELISA) was performed as described previously (18) with some modifications. The antigen used was a crude smooth LPS extract from *B. melitensis* 16M (the reference strain of biotype 1). The cells, grown and harvested as described above for *B. melitensis* 115, were extracted with hot water, and the extract was precipitated with 3 volumes of ethanol, dialyzed, and freeze-dried (18). Optimal conditions for antigen concentration and serum and conjugate dilutions were determined in preliminary experiments with the sera from 40 sheep culture positive for *B. melitensis* and 40 unvaccinated *Brucella*-free sheep. The antigen was suspended (2.5 μg/ml) in PBS, and it was dispensed (100 μl per well) in ELISA polystyrene microplates (Nunc A/S, Roskilde, Denmark). After overnight incubation at 37°C, the plates were washed with 0.05% Tween 20 in PBS (PBS-Tween) and then air dried. The dry plates were sealed and stored at 4°C, conditions under which they were stable for at least 4 months. The sera were diluted to 1:100 in PBS-Tween, 100 μl of this dilution was added to duplicate wells, and after incubation for 40 min at 37°C, the plates were washed three times with PBS-Tween before the addition of the conjugate (100 μl per well of a 1:3,000 dilution in PBS-Tween of polyclonal peroxidase-rabbit anti-sheep immunoglobulin [H+L specificity; Nordic Immunological Laboratories, Tilburg, The Netherlands]). The plates were incubated and washed as described above, and 100 μl of the substrate solution {0.1% ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt; Sigma], 0.004% H_2O_2, 0.05 M citrate buffer, pH 4} was added to each well. Optical density (OD) was determined at 405 nm in a Titertek Multiscan ELISA reader (Flow Laboratories, Ltd., Irvine, Ayrshire, Scotland) after 15 min of incubation at room temperature with continuous shaking. Reference positive and negative sera were included in all plates, and all sera were tested twice on the same day to assess repeatability.

The sensitivity of the tests was determined with the sera of 140 bacteriologically positive animals, and the specificity with the sera from the 100 control animals from *Brucella*-free flocks (see Results).

Animals. Two hundred ninety-one sheep were selected from five flocks in which *B. melitensis* had been previously isolated and no vaccination had been performed. All animals in the flocks were bled, tested in the RBT, and inoculated with Brucellergene (see the inoculation regimens described below); on the basis of the results of both tests, the animals were separated and classified into the following groups: (i) 210 sheep positive in both the RBT and DTH test, (ii) 68 sheep positive in only the DTH test, and (iii) 6 sheep positive in only the RBT. Seven sheep negative in both tests but belonging to the infected flocks were also selected (group iv). In addition, 100 unvaccinated sheep from a *Brucella*-free flock were used as controls.

DTH tests. (i) Evaluation of route, dose, time of reading, and type of allergen on the intensity of DTH reaction. One hundred eighty-four of the sheep positive in both RBT and DTH (group i) were divided into groups, and sheep in each group were inoculated with 0.1 ml of either CSA (50 or 100 μg in 0.15 M NaCl), cytosol (50 or 100 μg in 0.15 M NaCl), or Brucellergene (as provided by the manufacturer), both intradermally in the skin of the tail and subcutaneously in the right lower eyelid. The development of the DTH reaction was evaluated after 24, 48, 72 and 96 h by simple visual inspection and palpation and by measuring the skin thickness with a vernier-scaled caliper. In the first case, the test was considered positive when evident inflammation was present at the inoculation site. When the caliper was used, the test was considered positive when the skin thickness was increased by at least 20% over the value measured before inoculation.

(ii) Evaluation of the anergic state after first allergen inoculation. Concurrently with the above experiment, 107 of the 184 sheep in group i were divided in subgroups of 10 to 18 individuals and the subgroups were inoculated at regular intervals with the same dose and allergen as before but in the left lower eyelid. The development of a DTH reaction was assessed first visually and by palpation, and then by microscopic examination of samples taken at necropsy (see the description below).

Pathological and bacteriological examinations. Sheep were slaughtered and necropsied immediately after the results of the last DTH test were recorded. Samples taken from inoculation sites were fixed in Bouin's fluid for 24 to 48 h, included in paraffin, and processed and stained with hematoxylin-eosin by standard methods. Upon microscopic examination, those samples showing the infiltrates of macrophages and lymphocytes around dermis vessels characteristic of the DTH reaction were scored as positive.

Samples of spleen, milk (when available), uterus, and whole submaxillary, parotid, retropharyngeal, scapular, prefemoral, iliac, and mammary lymph nodes were also taken for bacteriological studies. All samples (with the exception of milk that was seeded directly) and lymph nodes were homogenized in small amounts of sterile saline (Stomacher LB80, Seward Medical, London, United Kingdom) and seeded on duplicate plates of both modified Thayer-Martin's (6) and Farrell's (13) media. The plates were incubated at 37°C in a 10% CO_2 atmosphere for at least 7 days, and colonies suspected of being *B. melitensis* were identified presumptively by Gram staining and oxidase and urease tests. Confirmation was carried out by phage and dye sensitivity tests, and typing of biovars was done by agglutination with monospecific A and M sera (3). The 140 strains isolated were identified as *B. melitensis* biovars 1 (81 strains) and 3 (59 strains).

Statistics. The comparison of percentages was carried out by the chi-square test, and mean comparisons were performed by *t* test.

TABLE 1. Influence of allergen, dose, inoculation site, and method of assessment of the reaction on the results of the DTH test

Allergen	Dose[a]	No. of animals	No. (%) of animals positive after inoculation in:			
			Eyelid		Tail	
			Visual[b]	Caliper[c]	Visual	Caliper
CSA	50	28	27 (96.4)	27 (96.4)	28 (100)	28 (100)
	100	24	24 (100)	24 (100)	24 (100)	24 (100)
Cytosol	50	20	20 (100)	20 (100)	20 (100)	20 (100)
	100	28	27 (96.4)	27 (96.4)	27 (96.4)	27 (96.4)
Brucellergene	0.1	84	84 (100)	84 (100)	83 (98.8)	83 (98.8)

[a] Doses are expressed in micrograms per animal for CSA and cytosol allergens and in milliliters per animal for Brucellergene.
[b] Results obtained by visual inspection and palpation of inoculation sites.
[c] Results obtained by measuring the skin thickness with caliper.

RESULTS

Characterization of allergens. The cytosol preparation contained 45.3% protein distributed in over 25 polypeptides ranging from apparent molecular weight of 72,000 to less than 14,400, as judged by SDS-PAGE and Coomassie-silver staining for proteins. By the same methods, CSA contained 53.5% protein and, although with both qualitative and quantitative differences, at least 15 polypeptides within the same apparent molecular weight range as those of the cytosol. SDS-PAGE of Brucellergene followed by protein staining revealed two protein bands (40K and 35K) which represented over 90% of the proteins detected and which were also present in the CSA and cytosol preparations. Periodate-silver staining of the gels did not detect the presence of rough LPS in any of the above extracts.

Evaluation of route, dose, and time of reading on DTH intensity. The DTH response was maximal after 72 h ($P < 0.05$ versus 48- or 96-h readings; data not shown), no matter the allergen, dose, or inoculation site, and no significant differences in the intensity of the reaction were observed among inoculation sites, doses tested (for CSA and cytosol), or allergens. Moreover, no significant differences were found in the DTH test results when the different allergens, doses, inoculation sites, and methods of assessment were compared (Table 1).

Anergic state induced upon inoculation with *Brucella* allergens. The results of these experiments are presented in Fig. 1. When tested 3 days after the first allergen inoculation, less than 20% of the animals developed a macroscopic DTH response. The percentage of reactors was 0% when the second inoculation was performed 6 days after the first, and it only

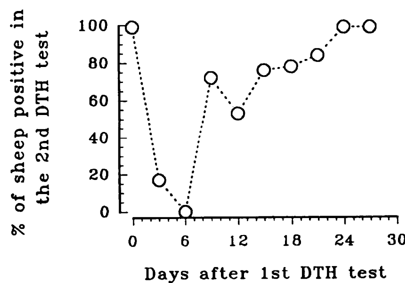

FIG. 1. Percentages of sheep giving a positive result in a second DTH test performed at different intervals after a first positive DTH test.

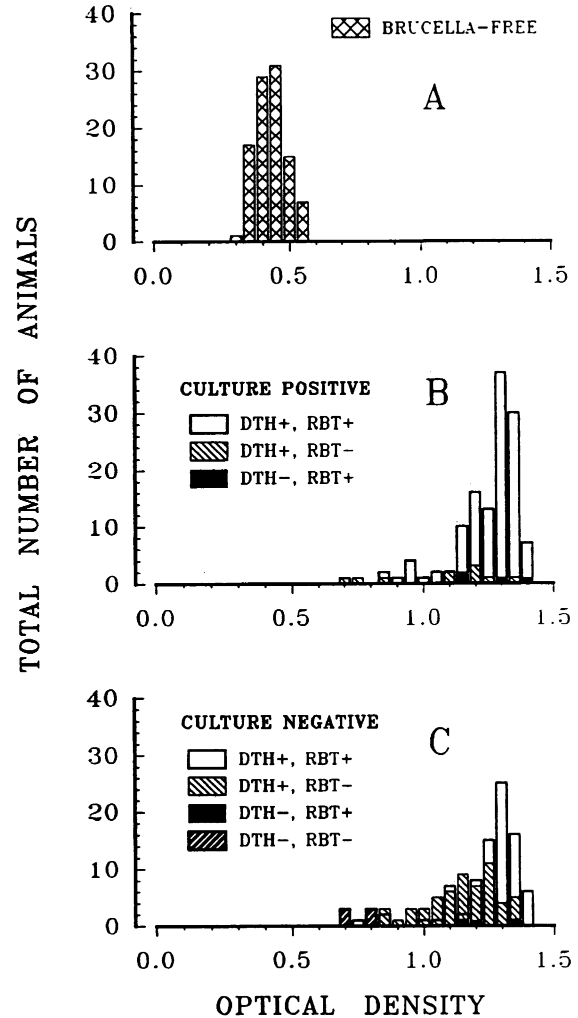

FIG. 2. Distribution of the ELISA OD (450 nm) values in the sera from 100 *Brucella*-free sheep (A), 140 sheep from which *B. melitensis* was isolated (B), and 151 sheep (belonging to infected flocks) from which *B. melitensis* was not isolated (C).

returned to 100% 24 days after the first inoculation (Fig. 1). However, even in the animals that were DTH negative by macroscopic criteria, microscopic examination of tissue samples showed infiltrates of macrophages and lymphocytes around dermis vessels, as well as the occasional presence of polymorphonuclear cells, suggesting an earlier antigen-antibody reaction.

Comparison of the results of bacteriological, serological, and DTH tests. Fig. 2 presents the ELISA results obtained with the sera from the 100 *Brucella*-free sheep (Fig. 2A) and from the 291 sheep in groups i to iv classified as culture positive (140 sheep; Fig. 2B) and culture negative (151 sheep; Fig. 2C) according to the results of the bacteriological search.

It can be seen that the sera from the *Brucella*-free sheep (mean OD ± standard deviation, 0.451 ± 0.06) were clearly discriminated ($P < 0.001$) from the sera of the sheep belonging to the infected flocks, no matter whether *B. melitensis* was isolated from them (Fig. 2B) or not (Fig. 2C). Moreover, the distribution of the ODs corresponding to the sera from the *B. melitensis* culture-positive sheep (mean OD ± standard deviation, 1.263 ± 0.14) was not different from that of the sera

TABLE 2. Results of the serological and DTH tests in culture-positive and culture-negative sheep from *B. melitensis*-infected flocks and in sheep from a *Brucella*-free flock

Flock and animal status	No. of animals	No. (%) of animals positive by:				
		RBT	CFT	ELISA	DTH determined:	
					Visually	Microscopically
Infected flocks						
Culture positive	140	129 (92.1)	124 (88.6)	140 (100)	136 (97.1)	140 (100)
Culture negative	151	87 (57.6)	81 (53.6)	151 (100)	142 (94.0)	144 (95.3)
Brucella free	100	0 (0)	0 (0)	0 (0)	0 (0)	ND[a]

[a] ND, not determined.

from the culture-negative sheep belonging to the infected flocks (mean OD ± standard deviation, 1.218 ± 0.16) (Fig. 2B and C). Notably, the seven sheep in group iv were culture negative and had positive ELISA results, with ODs ranging from 0.700 to 1.150 (Fig. 2C).

With the sera of the 140 bacteriologically positive sheep, the CFT was the least sensitive test, followed by the RBT (Table 2). There were two sera positive in the CFT and negative in the RBT. Thus, taken together, the CFT and RBT detected 131 of the 140 sera from bacteriologically positive animals (93.6%). In this same group of sheep, the ELISA identified as positive 100% of the sera and the DTH reaction assessed either visually or with a caliper was positive in 97.1% of the animals (Table 2). When the DTH reaction was assessed by a microscopic examination of the samples taken at necropsy from the inoculation sites, the sensitivity of the test reached 100% (Table 2).

In the 151 sheep from which *B. melitensis* was not isolated although they belonged to the infected flocks, the ELISA and the DTH test gave positive results for 100% of the sera and 94% of the animals, respectively (Table 2). On the other hand, the RBT and CFT identified as positive 57.6 and 53.6% of those sera, respectively (Table 2). None of the above-mentioned tests gave positive results for the 100 sheep from *Brucella*-free flocks (Table 2).

A comparison of the RBT and DTH results obtained in the group of 140 bacteriologically positive sheep showed that 125 sheep (89.3%) were positive in both tests, 11 sheep (7.8%) were positive in the DTH test and negative in the RBT, and 4 sheep (2.8%) were negative in the DTH test and positive in the RBT. In the group in which *B. melitensis* was not isolated, 84 sheep (56.3%) were positive in both the DTH test and the RBT, 57 sheep (37.7%) were positive in the DTH test and negative in the RBT, 2 sheep (1.3%) were negative in the DTH test and positive in the RBT, and 8 sheep (4.6%) were negative in both tests. In summary, there were more DTH-positive results than RBT positive results independently of the bacteriological status of the animals, although the differences were more conspicuous in the group of sheep from which *B. melitensis* was not isolated.

Finally, all the above-mentioned serological tests were repeated with sera taken from the allergen-inoculated animals immediately before slaughter, with results identical to those obtained before the first allergen inoculation.

DISCUSSION

DTH tests have been used for the diagnosis of sheep brucellosis with variable success (2, 14, 15, 23). The allergens used in early studies were generally obtained from culture supernatants or by acid extraction of *Brucella* cells (2) and, therefore, contained smooth LPS or its hydrolytic polysaccharides, which have undesirable effects. First, since the smooth LPS does not take part in DTH reactions (19, 20) and, in contrast, its O chain elicits a strong antibody response (10), injection of minimal amounts of smooth LPS in previously sensitized animals would cause an inflammatory reaction which could interfere with the interpretation of the skin test. Second, such extracts would cause a secondary antibody response interfering with future serological testing. Although in our experiments the microscopic examination revealed that mixed DTH-antibody-mediated reactions occurred occasionally at the sites where the allergens had been inoculated, these reactions cannot be due to antibodies to the smooth LPS. First, LPS was not detected in CSA, in Brucellergene, or in the cytosol. Moreover, since the LPS of *B. melitensis* 115 is devoid of the O-chain polysaccharide, the introduction by Jones et al. (20) of this strain in the preparation of allergens circumvents most of the problems pointed out above. In fact, the serum samples taken before allergen inoculation and at slaughter gave identical results in the ELISA with smooth LPS and also in the RBT and CFT, two tests which also detect antibodies to the smooth LPS (8). Therefore, the mixed reactions observed must be due to antibodies to the proteins present in the allergens, an interpretation consistent with the results of a previous study (30) which shows that a large proportion of sheep infected with *B. melitensis* develop antibodies to *Brucella* soluble proteins (30).

In agreement with previous reports, CSA had a complex protein profile (14, 20) and, indeed, this was also true of the cytosol. With quantitative differences, the three allergens shared the 40K and 35K polypeptides which were dominant in Brucellergene, and there were also qualitative differences. Since the intensity of the DTH response was similar for the three preparations, it is not possible to conclude whether polypeptides 40K and 35K are essential for DTH to *Brucella* allergens or, as the data suggest, a reduced proportion of them can be compensated for by other polypeptides. For diagnostic purposes, however, the results show that a purified protein would not offer advantages over complex protein mixtures. Because Brucellergene was used in the first screening for suspected animals, the possibility that CSA or cytosol would yield better results (in terms of sensitivity) than Brucellergene cannot be ruled out at present.

Most authors consider that the site and route of allergen inoculation is not important for DTH test sensitivity (2, 3, 15), and the method considered most efficient for sheep is the subcutaneous inoculation in the lower eyelid of 50 μg of CSA suspended in a 0.5-ml volume of saline, with readings taken 48 h after inoculation (3, 15, 21). Our results confirm most of those observations, but they also show that the intensity of the DTH responses peaked 72 h after inoculation. As discussed

above, mixed DTH-antibody-mediated intradermal reactions were occasionally observed. Since the antibody-mediated responses should be more apparent for shorter times, a reading time longer than 48 h also seems advisable for a better assessment of true DTH reactions.

To the best of our knowledge, an anergic response to *Brucella* allergens has not been documented before in the natural hosts. Anergy induced by repeated skin testing is a well-known phenomenon in bovine tuberculosis (28), and the results presented here show that DTH responses to *Brucella* allergens were lessened within the 24 days that followed a positive skin test. As expected, the lack of reactivity was not absolute and DTH reactions were evidenced when samples taken from inoculation sites after necropsy were examined microscopically. However, biopsy and microscopic examination of the samples would not be practical for diagnostic purposes, and the existence of the anergic state should be taken into account if the DTH test were to be used as a diagnostic test in the field.

It is generally acknowledged that DTH tests show no positive reactions (100% specificity) for *Brucella*-free flocks (2, 10, 15), and this is supported by the results obtained in our work with the 100 *Brucella*-free sheep. With regard to *Brucella*-infected flocks, there are few studies in which immunological testing is complemented with a bacteriological study. With small discrepancies, our results with the DTH test, CFT, and RBT (97.1, 88.6, and 92.1% sensitivities, respectively) and bacteriologically positive sheep bear an overall similarity to those of a study in which a *Brucella suis* allergen was used (11) and, for the serological tests, to those obtained by Trap and Gaumont (30). On the other hand, the RBT and CFT sensitivities found in our work are lower than those generally reported (for reviews, see references 2, 12, and 25). In most cases, this is because sensitivity was not defined with respect to the results of a bacteriological search, which is, however, the most objective criterion (1). In addition, the discrepancies could also relate to the sort of infection prevailing in the group of animals studied. In previous work (18), we found the CFT and RBT to be 100% sensitive when testing aborted sheep showing vaginal excretion of *B. melitensis*, and in the present work we have used mostly chronically infected sheep showing a mammary infection in over 80% of the cases (26). It can be hypothesized that the strong antigenic stimulation occurring in acute (abortive) infections would elicit high levels of antibodies to the smooth LPS (the immunodominant antigen involved in both RBT and CFT [8]), whereas in chronic infections bacteria within lymph node macrophages would not induce a strong serological response, with the ensuing decrease in RBT and CFT sensitivities. In any case, our results cast doubt on the value of the RBT as a screening test and the CFT as a confirmatory test for sheep brucellosis (2, 3, 25) and reinforce the conclusion that at least the standardization of both tests should be modified (5, 9).

Although the DTH test had a higher sensitivity than the RBT in the culture-positive animals and its use as a screening test in the field could be more practical than that of a serological test, 94% of the culture-negative animals belonging to infected flocks were DTH positive. The ELISA described here had an even higher sensitivity (100%), but it would present the same problems since it detected as positive 100% of the culture-negative animals belonging to infected flocks, including the animals in group iv that were also negative in the RBT and DTH test.

The high percentage of allergic and serological reactions in sheep from which *B. melitensis* was not isolated could be explained in two different ways. First, in infected flocks there could be a relatively large proportion of healthy animals which have repelled the infection by developing an immunological response. Second, it could be that the bacteriological procedures used were not sensitive enough to achieve the isolation of *B. melitensis* from all truly infected animals. Since the bacteriological methodology used has over 90% sensitivity in cattle (3), and the combination of the two selective media used in our work enables the detection of very low numbers of *B. melitensis* in infected sheep (26), we presently favor the first hypothesis. If proven correct, the existence of a relatively large proportion of animals positive in allergic and serological tests, but with no *B. melitensis* infection, suggests that removal of all positive-testing animals would unnecessarily increase the costs of eradication in endemic areas. Thus, it could be practical to develop indirect tests able to differentiate epidemiologically dangerous animals from those that are not.

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