JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1995, p. 706–712 Vol. 33, No. 3
0095-1137/95/$04.00+0

Specific Bovine Brucellosis Diagnosis Based on In Vitro Antigen-Specific Gamma Interferon Production

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Received 6 June 1994/Returned for modification 1 September 1994/Accepted 29 November 1994

In order to improve the specificity of the diagnosis of bovine brucellosis, we developed a test which can be regarded as an in vitro correlate of the delayed-type hypersensitivity test (DTH). A mixture of cytoplasmic proteins from *Brucella melitensis* B115 was used as a specific antigenic stimulus in bovine whole blood culture. Supernatants harvested at 18 to 24 h after the in vitro antigenic stimulus were assayed for their gamma interferon (IFN-γ) content by using a commercial sandwich enzyme-linked immunosorbent assay kit. The IFN-γ assay was evaluated with 10 heifers during the course (80 days) of an experimental infection and with 14 cows from an ongoing brucellosis outbreak. All of these animals were slaughtered, and pertinent organs were subjected to classical bacteriological analyses. In addition, we analyzed 23 field cases in which false-positive serological reactions occurred. The IFN-γ results were compared with those of the standard DTH and a battery of serological assays, and they were correlated with bacteriological data. Both for the experimental infection and for the field brucellosis outbreak, the IFN-γ assay detected infection in more animals than any combination of the serological tests, and it detected infection earlier than these tests. Finally, none of the samples from cows showing false-positive serological reactions was classified as positive by the IFN-γ assay, attesting to its specificity and to its usefulness in interpreting ambiguous serological results. A rapid and convenient alternative to the DTH, the IFN-γ assay appears to be an ideal method that is complementary to the serological diagnosis protocols.

The serological tests commonly used for brucellosis diagnosis are based on the detection of antilipopolysaccharide (anti-LPS) antibodies (2, 11, 26, 38). All have proved to be worthwhile for sanitary surveillance at the herd level. However, the multiplicity of the tests employed gives a clear picture of the inherent limitations of the serological approach (2, 3). In fact, no test on its own is able to detect both recently infected and more chronically infected animals (14, 34).

A more problematic aspect of serological detection emerged in the last 3 years in Europe: false-positive serological reactions (FPSR) either for cattle (3, 30) or for pigs (37). An FPSR is suspected when positive serological results in brucellosis diagnosis cannot be linked to any clinical or epidemiological data. Because of the test and slaughter sanitary regulations, such FPSR are of major concern. These FPSR are caused by the well-known antigenic relationships between *Brucella* LPS and the LPSs expressed by other gram-negative bacteria (8, 9). Among these, *Yersinia enterocolitica* O9 appears to be particularly important (1, 27), because its LPS O chain is almost identical to the O chain of *Brucella abortus* LPS and because it has been regularly isolated from feces of animals suspected of FPSR (30).

As an alternative to taking into account only the anti-LPS antibodies, it has been suggested that antibodies raised against selected *Brucella* proteins be tested for (25). In the case of humans and cattle, this goal has been approached either by Western blotting (immunoblotting) (7, 15, 39) or by enzyme-linked immunosorbent assay (ELISA) (7, 16, 24). From the growing amount of data and with the tests available, it appears that, compared with the anti-LPS response, the antiprotein antibody response elicited during brucellosis is far too heterogeneous and too delayed to be of great help in serological diagnosis (7, 23, 24).

Another way to circumvent the cross-reactivity caused by anti-LPS antibodies is to base the diagnosis on the specific cellular immune response. *Brucella* spp. are facultative intracellular pathogens inducing a cell-mediated immunity which, in mice, leads to the T-cell-dependent activation of macrophages through gamma interferon (IFN-γ) (19, 33). Several studies have demonstrated that the cell-mediated immunity can be used for the allergic diagnosis of bovine brucellosis by performing an intradermoreaction with *Brucella* protein extracts. This so-called delayed-type hypersensitivity assay (DTH) is extremely specific and is clearly complementary to the serological tests (4, 5, 13, 14).

Although very powerful at the herd level, the DTH is a rather tedious and time-consuming test, since animals have to be manipulated twice and the results are obtained after 2 or 3 days only.

An in vitro assessment of the specific cellular immunity should overcome these limitations, which are mostly caused by the in vivo nature of the DTH. In this way, quantification of the in vitro antigen-induced TH1 type of cytokine (e.g., IFN-γ) seems to be well correlated with the induction of a delayed-type hypersensitivity in vivo (20), and it has been successfully used by an Australian group as a diagnostic test for bovine tuberculosis (31, 35).

As IFN-γ appears to be an important mediator of acquired cell-mediated immune response also in a murine model of brucellosis (40, 41), it could be assayed for diagnostic purposes in the case of cattle. Therefore, the availability of a commercial

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sandwich ELISA kit allowing the detection of bovine IFN-γ prompted us to investigate the adequacy of this approach to brucellosis diagnosis.

In this paper we describe the use of an IFN-γ test in the context of bovine brucellosis diagnosis, the preliminary evaluation of this test compared with that of the DTH, and serological and bacteriological assays using both experimentally infected and naturally infected animals as well as the ability of the test to discriminate true brucellosis from FPSR.

MATERIALS AND METHODS

Infection of experimental animals. Ten brucellosis-free holstein heifers (six nonpregnant heifers and four in the first weeks of pregnancy) were infected via the conjunctival route with 6×10^7 viable cells of *B. abortus* 544. The animals were housed in a restricted area throughout the experiment.

A serological follow-up was scheduled at approximately 1-week intervals. Three successive DTHs were performed on days 35, 55, and 77. DTHs were not performed at earlier time points in order to avoid any additional in vivo sensitization of T cells which could interfere with the IFN-γ assay.

Blood samples for the IFN-γ assays were collected on days 0, 10, 25, 35, 55, and 77. All the animals were slaughtered on day 80, and a bacteriological examination was performed on selected organs and lymph nodes.

Field animals. In an ongoing brucellosis outbreak, 14 animals (holstein cows) were sampled for serology and IFN-γ testing on days 15 and 21 after the abortion by animal 4. Animals were slaughtered 1 day after the last sampling. A bacteriological examination was also conducted either on milk or on pertinent lymphoid tissues. At slaughter, this latter examination was used as an indication of infection.

Twenty-three animals belonging to 12 herds (both beef and milk cattle) and presenting FPSR were also selected for a comparison of the IFN-γ assay and the serological tests as well as for isolation of *Yersinia* spp. from feces.

Serological tests. The slow agglutination test in the presence of EDTA (EDTA-SAW), the complement fixation test (CFT), the rose bengal test (RB), and the ELISA were performed as previously described (26). The cutoff values of these tests are, respectively, 30 IU/ml, 20 IU/ml, any degree of agglutination, and 2.5 U/ml.

Milk tests. The milk ring test and the milk ELISA were performed according to the procedures described by Kerkhofs et al. (21).

Cellular assays. Cell-mediated immune response was investigated both in vivo by the DTH and in vitro by antigenic stimulation of whole blood cell cultures followed by an ELISA detection of IFN-γ.

(i) Antigenic preparation. The brucellin INRA is a mixture of cytoplasmic proteins from *B. melitensis* B115 (18). It was prepared by G. Dubray (Institut National de la Recherche Agronomique, Tours, France) and was used as a specific antigenic stimulus for the in vitro IFN-γ induction. The Brucellergene mixture (lot no. 96G091) is the commercial equivalent of the brucellin INRA. It was kindly provided by Rhone Merieux and was used for the DTH performed on experimental animals.

Both preparations have been extensively used to perform an allergic diagnosis of brucellosis (2, 11–14, 17, 18), and they were devoid of LPS as determined by Western blotting with anti-LPS monoclonal antibodies.

(ii) In vivo test: DTH. The DTH was performed by intradermally injecting 100 μl of Brucellergene (140 μg of proteins) into a previously shaved skin fold at the side of the neck. The thickness of the skin fold was measured with a spring skin meter (Aesculap, Tuttlingen, Germany) just before and approximately 72 h after the injection. A thickening greater than 1 mm was considered positive.

(iii) In vitro test: whole blood antigenic stimulation. Blood was collected from the jugular vein by using preservative-free sodium heparin as an anticoagulant. Samples were dispatched to the laboratory within 8 h. Stimulations were performed in duplicate by mixing in 24-well microplates (Nunc, Gand, Belgium) 1 ml of blood and either 100 μl of phosphate-buffered saline (PBS) (negative control) or antigenic stimuli (brucellin at 40 μg per well). The antigen was devoid of conservative agents and was diluted in 100 μl of PBS. The culture was incubated for 18 to 24 h at 37°C in a humidified atmosphere with 5% CO_2. Supernatants were then harvested and stored at −20°C until assayed for IFN-γ content.

(iv) IFN-γ assay. IFN-γ was assayed by using an ELISA kit (IDEXX, Paris, France). The test was performed according to the manufacturer's instructions.

Results were expressed in stimulation indices (SI) by using the following formula: mean of the optical densities of cultures with antigen divided by the mean of the optical densities of control cultures. A culture was considered to produce a significant level of IFN-γ if the SI was equal to or greater than the mean plus two standard deviations of the SI obtained for brucellosis-free control animals.

Bacteriological analysis. (i) Brucellosis studies. In the case of the experimental infection the following organs were selected: retropharyngeal, parotid, mandibular, suprascapular, supramammary, internal iliac, and mediastinal lymph nodes. If the heifers were pregnant, fetal envelopes as well as the fetal liver, lungs, and stomach content were also examined. For the field brucellosis outbreak, only the tonsils and the supramammary lymph nodes were used for bacteriological analysis.

At slaughter, all tissues were individually frozen at −20°C. Prior to bacterial culture, samples were completely thawed overnight. Ten milliliters of sterile PBS was added to 5 g of sample in a disposable bag which was then sealed and processed in a blender (Stomacher 80; Stuart Medical). A 100-μl portion of the homogenate was inoculated in duplicate on a solid selective Farel medium and incubated for 7 to 10 days in 5% CO_2 at 37.5°C. Identification and biotyping were performed according to standard procedures (2).

(ii) *Yersinia* sp. studies. In the herds for which FPSR were suspected, feces were collected and dispatched to the laboratory within 8 h. Fecal swabs were inoculated on *Yersinia* CIN (cefsulodin-Irgasan-novobiocin) plates (BioMerieux, Lyon, France) and incubated in a non-CO_2 incubator at 29°C for 24 to 48 h. Suspected or characteristic gram-negative colonies were plated on Trypticase soy agar (Becton Dickinson, Benelux) with 5% sheep blood. We used the BBL Crystal Enteric nonfermenter identification system (Becton Dickinson, Benelux). The biochemical identification was completed by antigenic analysis using *Y. enterocolitica* O:3- and O:9-specific antisera (Sanofi-Pasteur, Paris, France).

RESULTS

Development of the IFN-γ assay. In a preliminary stage, an animal experimentally infected with *B. abortus* 544 was selected on the basis of its reactivity both in an ELISA (8 U/ml) and in a lymphocyte blastogenesis assay in which the brucellin was used as an antigen (SI, 5) (data not shown). Heparinized blood from this animal was stimulated in vitro with increasing doses of brucellin (ranging from 1 to 100 μg per well) for 24, 48, or 72 h before harvesting and testing of the supernatant. The optimal parameters for IFN-γ production appeared to be a 24-h stimulation with a dose of 40 μg of brucellin per well. These parameters were used throughout the experimental protocol.

Several blood samples from 10 brucellosis-free control animals were tested with 40 μg of brucellin per well before the experimental infection. This precise dose was shown to produce an SI equal to 1.43 (1.43 ± 0.46) in the IFN-γ assay. On this basis, an SI was regarded as positive if it was equal to or greater than 2.5.

Evaluation of the IFN-γ test with experimentally infected animals. On the basis of the work of Limet et al. (26), both the conjunctival route and the dose were deliberately chosen to reproduce an infection leading to a weak seroconversion at the limit of detection of the officially approved serological tests. During the entire experiment, the animals were monitored by serological tests (CFT, EDTA-SAW, RB, and ELISA) and by cellular assays (DTH and IFN-γ) as described in Materials and Methods. For each test, the evolution of the number of positive animals starting at day 0 (the day of infection) and continuing to day 77 (three days before slaughter) is depicted in Fig. 1.

As far as officially approved European tests are concerned, the serological response is only transient. In fact, the CFT and the EDTA-SAW detected infection in three animals at day 25; only one of these remained positive from day 55 to the end of the experiment.

The RB detected infection in 2 animals as early as day 20, and it seems to have been far more efficient, as it also detected infection in 9 of the 10 animals at day 35. However, the serological evolution of the RB was still transient, because only one animal remained positive for the last two bleedings.

While the ELISA was less sensitive than the RB for the first three positive bleedings, the immune response measured by ELISA was more sustained than those measured by the classical serological tests, since among the 10 animals that tested positive at day 42, 9 remained positive until day 77. Even though these observations concern a limited number of animals, this kind of pattern is in close agreement with previously published data (26).

With regard to the cellular immune response, the IFN-γ

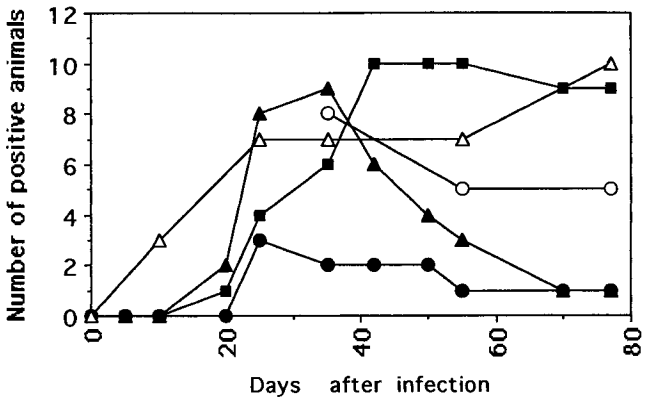

FIG. 1. Follow-up of the numbers of animals positive by the different tests during the experimental infection of ten heifers with *B. abortus*. Symbols: ●, CFT and EDTA-SAW; ▲, RB; ■, ELISA; ○, DTH; △, IFN-γ.

assay detected infection in three animals very early (at day 10), that is, more than 1 week before the earliest serological reaction. Once an animal became positive, it remained positive for all subsequent bleedings, and all the animals tested positive at the end of the experiment. Except for one cow at day 35, all animals positive by the DTH also tested positive by the IFN-γ assay. However, the DTH detected infection in only 5 animals in the last two skin tests, whereas, on the same dates, the IFN-γ assay detected infection in, respectively, 7 and 10 animals. Since the DTH was performed neither on day 10 nor on day 25, we cannot compare the two assays as far as early detection is concerned.

If we compare the immune statuses of these animals at day 77 with the bacteriological data obtained at slaughter (Table 1), the following comments can be made. Whereas 9 of the animals were negative by the classical serological tests, the ELISA and IFN-γ assay still detected infection in 9 and 10 animals, respectively. *B. abortus* was isolated from lymph nodes of 6 of these 10 animals. For five of these bacteriologically positive animals, *B. abortus* was recovered only from the lymphoid tissues near the inoculation site (parotid or mandibular lymph nodes). The nonpregnant animal, for which *B. abortus* was also isolated from a more distant site (a supramammary lymph node), was the only one which remained positive in all serological tests, and it gave the highest IFN-γ response. All 3-month-old fetuses were culture negative even if originating from a culture-positive heifer.

Evaluation of the IFN-γ test in an ongoing brucellosis outbreak. On a farm previously free of brucellosis, 1 animal (no. 4) among 14 animals aborted, and *B. abortus* biovar 3 was isolated from the milk, the genital tract, and the fetal products.

Because these animals were housed in a tie-stall barn, their respective locations remained unchanged during the sampling period. This allowed us to monitor the dissemination of the infection, by horizontal spreading, from the diseased animal to the neighboring cows. Tables 2 and 3 summarize the results obtained at 6 days and 1 day before the slaughter of the 14 animals (i.e., at 15 and 21 days, respectively, after the abortion by animal 4).

At 6 days before slaughter (Table 2) only the animal which aborted (cow 4) was classified as positive by serology whatever the test used. This cow was also the only one detected by the milk ring test. With regard to the milk ELISA, it allowed the detection of both cows 3 and 4. At the same time three animals (no. 2, 3, and 4) were classified as positive according to the IFN-γ results, whereas no excretion of *B. abortus* in the milk could be detected by classical bacteriological methods.

At 1 day before slaughter (Table 3) four animals (no. 3, 4, 5, and 6) appeared to be positive by the serological tests interpreted in parallel, while cows 2, 3, 4, 5, 7, and 10 were detected by the IFN-γ assay.

At slaughter, *B. abortus* biovar 3 was isolated either from the tonsils or from the supramammary lymph nodes of animals 2, 3, 4, 5, 6, 7, and 12. This last cow probably illustrates the very early stage of the infectious process, since it was infected without having yet mounted a detectable immune response. The majority of the infected animals, defined as positive by the bacteriological analysis, were located in the immediate vicinity of the animal which aborted. All of the remaining animals were found to be negative by bacteriological analysis. The reasons why no *B. abortus* was recovered from animal 10, which was

TABLE 1. Comparison of the bacteriological data and the results of serological and cellular assays at day 77 following the experimental infection of 10 heifers with *B. abortus*

Animal	Result of:							
	Serology[a]				Cellular assay		Bacteriological analysis	
	EDTA-SAW (IU/ml)	CFT (IU/ml)	RB (degree of agglutination)[b]	ELISA (U/ml)	DTH (Δ cm)[a,c]	IFN-γ (SI)	Cow	Fetus
1	400	1,600	4+	>60	0.16	>20	+[d]	
2	−	−	−	7.2	0.14	10	+[e]	
3	−	−	−	12.7	0.21	10	+[e]	
4	−	−	−	3	−	8.5	+[e]	
5[f]	−	−	−	2.7	−	6.7	+[e]	−
6[f]	−	−	−	3.8	−	9.7	+[e]	−
7[f]	−	−	−	8.8	0.17	8.1	−	−
8	−	−	−	12.4	0.15	5	−	
9[f]	−	−	−	−	−	4.6	−	−
10	−	−	−	3.1	−	10	−	

[a] Values under the respective thresholds for the serological and DTH tests are indicated by minus signs.
[b] Degree of agglutination was scored from + to 4+ according to the intensity and rapidity of the reaction.
[c] Change in thickness of skin fold (see Materials and Methods).
[d] *Brucella* organisms isolated from mandibular, supramammary, and internal iliac lymph nodes.
[e] *Brucella* organisms isolated from mandibular or parotid lymph nodes.
[f] Pregnant heifer.

TABLE 2. Humoral and cellular follow-up of an ongoing brucellosis outbreak[a]

Animal[b]	Result for:			
	Serum		Blood (IFN-γ test [SI])	Milk
	Classical tests	ELISA (U/ml)		
1	−	−	−	−
2	−	−	3	−
3	−	−	9	+[c]
4[d]	+	>60	>20	+[e]
5	−	−	−	ND
6	−	−	−	−
7	−	−	−	−
8	−	−	−	−
9	−	−	−	−
10	−	−	−	−
11	−	−	−	ND
12	−	−	−	ND
13	−	−	−	−
14	−	−	−	−

[a] Fourteen animals were sampled for serology and for milk and IFN-γ testing at 15 days after the abortion of animal 4.
[b] Numbers correspond to animals' locations in the barn.
[c] Milk ELISA positive only.
[d] Abortion with *B. abortus* isolation (see the text).
[e] Milk ELISA and milk ring test positive.
[f] ND, not tested (no secretion).

TABLE 3. Humoral and cellular follow-up of an ongoing brucellosis outbreak[a]

Animal[b]	Result			
	Serum		Blood (IFN-γ test [SI])	Bacteriological analysis
	Classical tests	ELISA (U/ml)		
1	−	−	−	−
2	−	−	4	+[c]
3	−	6	15	+[d]
4	+	>60	>20	+[d]
5	+	4	9	+[d]
6	+[e]	−	−	+[d]
7	−	−	6	+[c]
8	−	−	−	−
9	−	−	−	−
10	−	−	5	−
11	−	−	−	−
12	−	−	−	+[d]
13	−	−	−	−
14	−	−	−	−

[a] The animals used for Table 2 were sampled again for serology and IFN-γ testing 6 days later and submitted to bacteriological studies at slaughter.
[b] Numbers correspond to animals' locations in the barn.
[c] *B. abortus* isolated from tonsils.
[d] *B. abortus* isolated from supramammary nodes.
[e] All the classical tests except CFT were positive.

positive by the IFN-γ test, might be linked to the inherent limitations of the method used (e.g., the type of organ or the sensitivity of the bacteriological tests).

Use of IFN-γ to discriminate true brucellosis from FPSR. When FPSR are suspected in officially approved serological tests, the animals concerned are usually submitted to a battery of serological tests (including ELISA) at 2-week intervals, as well as to a bacteriological analysis of the feces. Twenty-three such animals in 12 herds were selected. Their serological data, compared with the results of the IFN-γ assay, are illustrated in Table 4, as are the results of the *Y. enterocolitica* O:9 isolation from the feces.

Among the animals tested, none was positive for IFN-γ, whereas they all showed, on the same day, an ELISA titer in the range of 3 to 60 U/ml. *Y. enterocolitica* O:9 was isolated from the feces of five animals.

According to the subsequent clinical and serological survey, none of these animals turned out to be a true brucellosis case and all of them returned to a negative serological status or at least to minimal titers within 12 weeks. With this limited trial, the specificity of the IFN-γ test was maximal.

DISCUSSION

Perhaps more than other intracellular bacteria, *Brucella* species induce an antibody response which is commonly used as a diagnostic tool. The detection of anti-LPS antibodies is, up to now, most convenient for this purpose (2, 34, 38). Nevertheless, the close antigenic relationship between the LPS of *B. abortus* and that of *Y. enterocolitica* O:9 (8, 9) leads to frequent FPSR (30). While some of the FPSR can be abolished by the use of modified tests (i.e., tests incorporating seroagglutination in the presence of EDTA [28] or pretreatment of the serum with a reducing agent before the agglutination process [22]), most of them cannot be circumvented by the combined use of a panel of serological tests.

It has been claimed that the search for cell-mediated immunity in bovine brucellosis can be helpful in resolving the diagnostic dilemma linked to serology (6, 14). In fact, results of the DTH with proteins extracted from *Brucella* species were clearly proven to be complementary to serological data (4, 5, 7, 12). Moreover, the DTH was also known to be one of the most specific tests, thus allowing one to solve the problem of the remaining ambiguous false-positive reactions (14, 17).

However, as a consequence of its in vivo realization, the DTH suffers from several limitations: 2 to 3 days is required to obtain the results (2, 14); the animals have to be manipulated twice; and the intensity of the DTH reaction is usually limited (e.g., one-third to one-fourth of the intensity reached by a tuberculin test) (31a), leading to difficulties in interpreting the results. Finally, the DTH, when repeated, can lead to modification of the immune status of the animal.

In vitro techniques for assessing the cellular immune response are devoid of such drawbacks. Yet, some of them, such as the T-cell proliferation assay, while able to discriminate brucellosis from *Y. enterocolitica* infection (6), require some technical hints and expertise (i.e., for cell isolation and radioactivity incorporation), and they still require several days for the recovery of data.

In the context of tuberculosis, the quantification of IFN-γ produced in a whole blood cell culture following the response to an antigen-specific stimulus is free of these disadvantages. This technique is not only far more rapid and simple than the DTH or the proliferation assay, but it also appears to be more sensitive (31, 35).

The IFN-γ assay was evaluated for bovine tuberculosis diagnosis during a large field survey in Australia, and it demonstrated a specificity of 96% and an increase in sensitivity compared with the usual DTH (36).

The availability of a commercial ELISA kit for the quantitation of bovine IFN-γ prompted us to build a diagnostic test in the context of bovine brucellosis, using *Brucella* proteins as an antigenic stimulus in vitro. The rationale for the choice of brucellin as the antigen to stimulate whole blood cell culture was the large amount of data concerning this preparation as

TABLE 4. Comparison of IFN-γ assay results and serological or bacteriological data for confirmed FPSR cases

Animal[a]	Result					
	Serology				IFN-γ assay (SI)	Bacteriological analysis (organism detected)
	EDTA-SAW (IU/ml)	RB (degree of agglutination)[b]	CFT (IU/ml)	ELISA (U/ml)		
LO 1	25	2+	15	30	0.9	*Y. enterocolitica* O9
LO 2	25	−	10	4	1.4	
LO 3	50	2+	100	30	1.0	
LO 4	12.5	−	0	16	1.3	
LO 5	25	−	ND[c]	16	0.9	
VE 6	50	−	15	3	0.8	
DE 7	50	2+	50	43	0.8	
DI 8	25	+	25	40	1.2	
JO 9	12.5	ND	30	30	2.3	
FE 10	50	3+	15	ND	1.5	
MA 11	50	3+	>100	55	1.0	
MA 12	100	4+	>100	>60	1.5	
ME 13	25	1+	15	8	1.5	
ME 14	25	2+	0	8	2.4	
ME 15	25	4+	0	8	1.4	
ME 16	25	2+	0	8	1.4	
BE 17	25	1+	15	4	1.6	*Y. enterocolitica* O9
MT 18	25	3+	0	8	1.2	
MT 19	25	1+	10	16	1.6	
MT 20	25	2+	10	16	2.4	
ZE 21	50	ND	50	57	1.3	*Y. enterocolitica* O9
ZE 22	50	ND	25	25	1.0	*Y. enterocolitica* O9
ZE 23	25	ND	20	20	1.0	*Y. enterocolitica* O9
Total[d]	35	79	41	100	0	

[a] The two-letter codes in front of the numbers refer to the different herds evaluated.
[b] Degree of agglutination was scored from 1+ to 4+ according to the intensity and rapidity of the reaction.
[c] ND: not determined.
[d] Percent positive animals for each test according to the number of animals tested.

well as its current use in the DTH (13, 17, 18). In order to evaluate the ability of the IFN-γ assay to detect infection in positive animals early in the course of infection and to evaluate its relative sensitivity, compared with those of other tests, either humoral or bacteriological, we used both an experimental infection and a natural infection.

In the context of the experimental infection of nonpregnant heifers (or animals at an early stage of pregnancy), the IFN-γ assay was shown to detect infection earlier than serological tests (even RB) and to be more sensitive than classical serology. The comparison with the ELISA requires some more comments: while the ELISA detected infection in all the animals from day 47 onwards (nine of them remaining positive for the two last bleedings), the IFN-γ test revealed infection in seven animals from day 25 to day 55. This discrepancy cannot be linked to the suspected impact of a strong serological response on the cellular immune response (taking the DTH as a model) (29). In fact, except for one animal which reached a high ELISA titer (>60 U/ml) at the last three bleedings and was still positive by the IFN-γ assay, all the cows showed rather low ELISA titers (never exceeding 15 U/ml). Concerning the abrupt rise (from 7 to 10) in the number of animals that tested positive by the IFN-γ assay on the last sampling date (day 77), we cannot entirely preclude the sensitization effect which could have resulted from repeated DTHs in the case of the last IFN-γ test only. However, with the allergen used, such an effect on the humoral immune response as measured by the anti-LPS ELISA can be ruled out without any doubt (13). In our experiment, the anti-LPS ELISA titers were not influenced by the three successive DTHs (data not shown).

While detecting the highest proportion of infected animals, the IFN-γ assay was not able to detect all of the infected animals throughout the experimental infection. However, the combined use of this test with the serological screening allowed the detection of 90% of the animals as early as 25 days after infection and 100% of them starting at day 35.

Although all animals received the same dose of virulent bacteria via the same route, *B. abortus* was successfully isolated from the lymph nodes close to the inoculation site in the case of only 6 of the 10 animals, and for one of these, it were isolated from the supramammary lymph nodes also. This shows the need to carefully select the organs sampled for bacteriological analysis and points out the fact that classical bacteriology cannot always be taken as a reference for the estimation of the sensitivities and specificities of other diagnostic methods.

Concerning the follow-up of the horizontal spreading of an infection after an abortion, the overall conclusions paralleled those made for the experimental infection. The IFN-γ assay usually became positive before the serological tests, and it detected infection in the largest number of animals. This assay still remained complementary to the other tests (see the results for animal 6 [Table 3], which was classified as positive only by serology).

Finally, the IFN-γ assay is very powerful in its ability to discriminate true brucellosis from FPSR. This was demonstrated with 23 cows which illustrated the commonly encountered serological diagnosis dilemma that occurs when the clinical and epidemiological data for brucellosis are missing. All of these animals remained negative by the IFN-γ assay. Because of the fact that no brucellosis was declared to be present in the

herds concerned and since all the animals returned to a negative serological status or to minimal titers within 12 weeks, the specificity of the IFN-γ assay appears to have been excellent.

The cutoff for the IFN-γ assay was determined, in a preliminary step, by using prebleedings of the 10 heifers used for the experimental infection, and it was fixed at an SI of 2.5. As far as the cases of FPSR reported above are concerned, this cutoff value seems to be well adapted. When we performed an IFN-γ assay on 40 animals (of various ages) from herds officially free of brucellosis and of FPSR for at least 5 years, none of these animals was classified as positive (data not shown). Taking into account the data obtained for the 10 experimental animals (before infection), for the 23 cases producing FPSR, and for the 40 above-mentioned animals, the specificity of the IFN-γ assay is 100%. Future validation of this cutoff and the determination of the exact specificity of the IFN-γ assay will require more extensive field trials.

The presence of LPS in the antigenic preparation used for the induction of IFN-γ could be detrimental to the specificity of the test by activating the natural arm of the cellular immune response (i.e., by activating NK cells either directly or indirectly via macrophages through interleukin 12 synthesis) as described previously for humans (10). In fact, in our study, purified LPS or even intact *Brucella* smooth cells induced an IFN-γ synthesis even in animals free of brucellosis (data not shown), suggesting the same nonspecific stimulation. The allergen used for the IFN-γ test was devoid of LPS, as confirmed by Western blotting with anti-LPS monoclonal antibodies, and it did not induce nonspecific IFN-γ production when tested on animals free of brucellosis. Nevertheless, as one would expect, the cytoplasmic proteins contained in the brucellin could well be not strictly specific for the genus *Brucella*, and they could probably recall in vitro some memory T cells specific for related proteins of other genera, leading to a limited production of IFN-γ. This could be one of the reasons why, in our study, some animals free of brucellosis had an SI in the IFN-γ assay that was close to the cutoff (i.e., reaching 2.4).

Like the other tests available, the IFN-γ assay is probably not able to distinguish vaccinated animals from infected animals. Nevertheless, the fact that it can both detect an infectious process very early in time and detect infection in a high proportion of infected animals leads to the conclusion that this test can significantly contribute to the existing eradication program for bovine brucellosis.

Furthermore, because of its potency for solving the problems associated with FPSR, this test is nowadays used in the field as a confirmation test both in France (14a) and in Belgium when FPSR are suspected for cattle. Mutatis mutandis, a similar IFN-γ assay could also be applied to other species for which FPSR perturb brucellosis diagnosis (32).

ACKNOWLEDGMENTS

V. Weynants and J. Godfroid contributed equally to this work. We thank Dominique Gilson and the staff of Machelen and Erpent for providing their technical support. We also thank D. Prozzi for correcting and improving our English.

This work was supported by a grant from the Ministère de la Région wallonne pour l'Agriculture (no. 2285) and a grant from the Ministère Belge de l'Agriculture (PDZR grant).

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