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Evaluation of PCR and Indirect Enzyme-Linked Immunosorbent Assay on Milk Samples for Diagnosis of Brucellosis in Dairy Cattle

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A study was performed to evaluate the previously described PCR (C. Romero, C. Gamazo, M. Pardo, and I. López-Goñi, J. Clin. Microbiol. 33:615–617, 1995) for the diagnosis of brucellosis in dairy cattle. Milk samples from 56 *Brucella* milk culture-positive cattle and from 37 cattle from *Brucella*-free herds were examined for *Brucella* DNA by PCR and for specific antibodies by an indirect enzyme-linked immunosorbent assay (ELISA). The specificities of both tests were 100% when testing the milk samples from *Brucella*-free cattle. The milk samples from 49 infected cattle were positive by PCR (87.5% sensitivity), and 55 were positive by ELISA (98.2% sensitivity). A PCR-positive sample was negative by ELISA, and 7 ELISA-positive samples were PCR negative, yielding an observed proportion of agreement of 0.91 for the two tests. Although the results suggest that ELISA is a better screening test than PCR, the combined sensitivity of the two assays was 100%, and their simultaneous application could be more useful than one test alone for a rapid screening of brucellosis in dairy cattle.

The most certain test for an accurate diagnosis of brucellosis is the bacteriological isolation of *Brucella* spp. (2). In dairy cattle, milk samples and selective media are used most often (2). However, when testing large numbers of cattle, this direct diagnostic test is often impractical and indirect tests detecting antibodies in serum or milk are used routinely to screen for cattle suspected of being infected. The milk ring test is probably the most widely used test for the screening and monitoring of brucellosis in dairy cattle (2). Although the sensitivity of the milk ring test is satisfactory (14), its specificity has been questioned when prevalence is low, and additional serological tests such as several indirect enzyme-linked immunosorbent assays (ELISA) have been developed and successfully tested on milk samples (23). However, antibody detection is not wholly satisfactory because not all infected animals produce significant levels of antibodies and several bacteria can produce cross-reacting antibodies (2). Because of these difficulties, the development of new diagnostic tests for the direct detection of *Brucella* spp. in blood, milk, or other samples is increasingly interesting.

PCR assay has been shown to be a valuable method to detect DNA from different microorganisms. Although there are several studies of *Brucella* DNA detection by PCR with pure cultures (3, 11, 13, 20), few studies have been performed with clinical or field samples (10) and no comparisons with bacteriological and serological tests have been made. The aim of this study was to compare a previously described PCR assay (20) with bacteriological and serological tests with milk samples for the diagnosis of bovine brucellosis.

MATERIALS AND METHODS

Milk samples. Milk was obtained from 56 dairy cows infected in the field as determined by the isolation of *Brucella* spp. All of these animals tested positive by the standard rose bengal and complement fixation tests for brucellosis (2). In addition, milk samples were collected at random from 37 cattle from two brucellosis-free dairy herds. Sera from milk were obtained by incubating 10 ml of milk with 5 drops of rennet (1:10,000) at 37°C overnight and centrifuging the sample at 6,000 × *g* for 15 min. Milk samples to be used in the bacteriological assay were stored at 4°C, while milk for the PCR and milk sera for the ELISA were stored at −20°C.

Bacteriological examinations. The cream and sediment obtained after centrifugation (3,000 × *g*, 15 min, 4°C) of 50 ml of milk were seeded on duplicated plates of both Farrell's (2, 9) and modified Thayer-Martin's (6) media, a method which increases sensitivity for the isolation of brucellae (17). The plates were incubated in a 10% CO_2 atmosphere at 37°C for at least 7 days, and suspected colonies were identified by colonial morphology, Gram staining, and oxidase and urease tests (2). Typing of the isolates was performed by phage and dye sensitivity tests and agglutination with monospecific antisera according to standard procedures (2).

Sample processing for PCR. In preliminary experiments, we examined the following parameters to optimize DNA extraction: the composition of the extraction buffer, the previous heating step, RNA digestion at different steps, and the use of commercial DNA purification resins (InstaGene matrix; Bio-Rad Laboratories, Madrid, Spain). The best results were obtained under the conditions described below.

Frozen milk was thawed at room temperature, and 500 μl of the sample was mixed with 100 μl of 50 mM NaCl–125 mM EDTA–50 mM Tris-HCl (NET) (pH 7.6). After incubation at 80°C for 15 min, the mixture was cooled on ice for 2 min and digested with RNase (ICN Pharmaceuticals Inc., Irvine, Calif.) at 50°C for 1 h. Sodium dodecyl sulfate (SDS; 0.5%) and proteinase K (200 μg/ml) were added, and the mixture was incubated at 50°C for 3 h. Cell debris was removed by precipitation with 5 M NaCl and a hexadecyltrimethylammonium bromide-NaCl solution at 65°C for 10 min (24). DNA was extracted by the standard protocol with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, washed with 70% ethanol, and dried under a vacuum (22). The DNA pellet was resuspended in 25 μl of sterile distilled water and stored at −20°C until further use. One microliter of this DNA suspension was added to the PCR cocktail reaction mixture. Alternatively, DNA extraction was performed without previous RNA digestion, and the final 25 μl of DNA suspension was digested with RNase at 37°C for 1 h. Each experiment included one sample of sterile milk inoculated with a suspension of *B. abortus* 2308 and one sample of sterile milk as the internal positive and negative controls, respectively.

Amplification and detection of *Brucella* DNA by PCR. The oligonucleotide primers used were selected from the 16S rRNA sequence of *B. abortus* previously published (8). A 905-bp fragment was amplified with primers F4 and R2 as described before (20) with minor modifications. Briefly, PCR was performed in a 25-μl volume containing the reaction mixture and acetylated bovine serum albumin (Promega Biotec, Madison, Wis.) to a final concentration of 200 μg/ml to block enzyme inhibitors. The reaction mixture was processed in a Gene ATAQ Controller System (Pharmacia Biotech, S.A., Barcelona, Spain). The cycling conditions consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 54°C for 90 s, and 72°C for 90 s and a final extension at 72°C for 6 min. The positive control contained 80 ng of *B. abortus* 2308 DNA as the template, and the negative control consisted of sterile water instead of the DNA template. Generally recommended procedures were used to avoid contamination (19). After amplification, 7 μl of the reaction mixture was electrophoresed on an 0.8% agarose gel, stained with ethidium bromide, and photo-

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TABLE 1. Results of culture, PCR, and indirect ELISA tests with milk samples from cows belonging to infected herds

Culture type	No. of samples	PCR		ELISA	
		Positive	Negative	Positive	Negative
B. abortus biovar 1	10	9	1	10	0
B. abortus biovar 3	13	12	1	13	0
B. melitensis biovar 3	31	26	5	30	1
Brucella spp.[a]	2	2	0	2	0
Total	56	49	7	55	1

[a] These isolates could not be typed.

TABLE 2. Comparison of PCR and indirect ELISA results for milk from cows from infected herds and from *Brucella*-free cattle

PCR result	No. of animals	
	ELISA positive	ELISA negative
Positive	48[a]	1[a]
Negative	7[a]	37[b]
Total	55	38

[a] All of these samples were culture positive.
[b] All of these samples were culture negative.

graphed on a UV transilluminator. The presence of a clear-cut band of 905 bp was regarded as a positive result. Each sample was tested at least in triplicate.

PCR limit of detection in inoculated milk. Sterile bovine milk was inoculated with either *B. abortus* 2308 or *B. melitensis* 115, and the actual number of CFU per milliliter of milk was determined by plate counting on tryptic soy agar. Aliquots of 0.5 ml were extracted and processed by PCR as described above. All experiments were run in triplicate.

ELISA. An indirect ELISA was performed as described by Díaz-Aparicio et al. (7) with some modifications. The antigen was a smooth lipopolysaccharide-rich extract obtained from *B. melitensis* 16M. Two different conjugates were used: a commercial peroxidase-recombinant protein G (Pierce Chemical Co., Rockford, Ill.) and a polyclonal (rabbit) anti-bovine immunoglobulin A (Miles Laboratories, Inc.) conjugated with horseradish peroxidase type VI-A (Sigma Chemical Co., St. Louis, Mo.) by the method described by Nakane and Kawaoi (18). Milk sera from a healthy and an infected animal were used as negative and positive controls, respectively.

Statistics. For each test, the sensitivity (the absence of false-negative results), specificity (the absence of false-positive results), and predictive value (the probability of a true diagnosis, no matter whether positive or negative) were calculated with the Episcope software (K. Frankena and J. O. Goelema, Agriculture University, Wageningen, The Netherlands) with a 95% confidence level. The proportion of observed test agreement was also calculated.

RESULTS

PCR limit of detection in inoculated milk. To assess the limit of detection (expressed as CFU per milliliter) of our PCR assay in milk, sterile bovine milk was inoculated with a known number of either *B. abortus* 2308 or *B. melitensis* 115 organisms and processed subsequently for PCR amplification and culture. Under these conditions, the PCR limit of detection corresponded to a mean of 170 CFU/ml for *B. abortus* 2308 and 1,700 CFU/ml for *B. melitensis* 115.

Comparison of culture and PCR. Of the 56 *Brucella* strains isolated from milk, 23 were identified as *B. abortus* (10 from biovar 1 and 13 from biovar 3), 31 were identified as *B. melitensis* biovar 3, and 2 could not be typed into species and biovar. Of these 56 culture-positive samples, a total of 49 were positive by the PCR test (Table 1). On the other hand, all the samples from the 37 *Brucella*-free cattle were negative by PCR. Accordingly, the sensitivity of PCR with respect to bacteriological culture was 87.5%, and the specificity was 100%. The predictive value of a positive test result was 100%, and the predictive value of a negative test result was 84.1%.

The presence of PCR-interfering substances which could account for the PCR-negative results for the culture-positive samples was examined in two of these samples. One of them yielded a PCR-positive result only after a 10-fold dilution. The other was PCR-negative even when processed after being mixed with a suspension of 10^9 CFU of *B. abortus* 2308 per ml.

Comparison of culture and ELISA. With peroxidase-protein G as the conjugate, only 1 of the 56 bacteriologically positive samples was ELISA negative (Table 1). A negative result was also obtained when this sample was tested for anti-*Brucella* immunoglobulin A by ELISA. These results mean a sensitivity of 98.2% for the indirect ELISA when compared with the bacteriological culture. The predictive value of a positive ELISA result was 100%, and the predictive value of a negative result was 97.4%. No anti-*Brucella* antibodies were detected by ELISA with protein G or anti-immunoglobulin A conjugates in the serum of the 37 milk samples from *Brucella*-free animals (100% specificity).

Comparison of PCR and ELISA. When PCR and ELISA results were compared (Table 2), coincident results were obtained in 85 of the 93 samples (48 were positive and 37 were negative by both tests), yielding an observed proportion of agreement between PCR and ELISA of 0.91. The seven PCR-negative culture-positive samples (see above) gave positive results in the ELISA, and the culture-positive sample which was ELISA negative (see above) was positive by PCR.

DISCUSSION

In a previous work (20), the PCR oligonucleotide primers were selected from the 16S rRNA sequence of *B. abortus* and their conditions of use and specificities were evaluated with DNA from bacteria phylogenetically and serologically related to *Brucella* spp., from clinical isolates of non-*Brucella* species, and from the representative strains of *Brucella* biotypes. In this study, we have evaluated this PCR protocol for detecting *Brucella* DNA in bovine milk samples.

The results with inoculated milk with known numbers of *Brucella* organisms showed that the limit of detection for *B. abortus* 2308 viable cells by the proposed PCR assay was 170 CFU/ml, and for *B. melitensis* 115 the detection limit was 10-fold concentrated. However, in our previous work (20), the threshold sensitivities of the PCR assay determined by testing serial DNA dilutions were similar for *B. abortus* 2308 and *B. melitensis* 115 (50 to 60 fg of DNA, corresponding to approximately 15 to 20 cells). These results suggest that the different limits of detection for cells of *B. abortus* 2308 and *B. melitensis* 115 may be due to differences in the effectiveness of the DNA extraction protocol. Also, although no CFU determinations could be performed routinely on the milk from cattle infected in the field, PCR-positive results were often observed for milk samples containing fewer CFU per ml than those inoculated milk samples determined to be at the limit of detection. These observations also suggest that factors such as sample storage and handling, particular *Brucella* strain, etc., could affect the final outcome of the assay by affecting DNA release. Further research is necessary to clarify these factors which could be limiting the diagnostic sensitivity of the PCR.

When the PCR was applied to field samples, its sensitivity with respect to bacterial culture was 87.5%. The presence of polymerase inhibitors (19) could account, at least in part, for a PCR-negative result in samples that were culture positive. This interpretation is supported by the analysis of two samples which gave discrepant results and were available for further study. In both, the additional results strongly suggested the

presence of inhibitors. Many substances have been suggested to be amplification inhibitors, including hemoglobin, urine, heparin, phenol, and SDS (12, 15). Further studies will be necessary for the identification of other inhibitory factors. Other hypotheses to account for those false-negative PCR results are a number of organisms below the detection limit, the degradation of target DNA in the samples, and inefficient DNA extraction.

Because of its specificity, the bacteriological culture is regarded as the gold standard in the evaluation of indirect tests, such as immunological tests (1), which can be positive without the actual presence of the causative organism. However, it is not known whether bacteriological culture has the same value in the evaluation of tests which specifically detect the presence of bacterial molecules. In fact, in preliminary experiments, we have found PCR-positive results in animals which belong to infected herds and show positive results by serological tests while being milk culture negative. A false-negative bacteriological result can be caused by a massive contamination of the milk samples, by the inhibition of some *B. melitensis* strains in the selective medium (4), or by a viability loss before culturing, and in all these circumstances DNA can still be detected by PCR.

Under our conditions, the ELISA with protein G showed excellent sensitivity (98.2%) with respect to culture, and its specificity was 100% when testing milk from *Brucella*-free cattle. These results support the findings of other authors on the value of the indirect ELISA as an alternative for the individual screening of brucellosis when milk is available (5, 16, 21). Although the ELISA with protein G showed a better sensitivity than PCR, a culture-positive animal was ELISA negative and PCR positive and all infected animals were detected only when the results of both tests were considered. Thus, the results presented here show that the complementary use of ELISA and PCR as rapid screening tests shows considerable promise for the diagnosis of brucellosis in dairy cattle.

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