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Rapid Identification of Smooth *Brucella* Species with a Monoclonal Antibody

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A colony blot enzyme-linked immunosorbent assay was developed for the rapid identification of smooth *Brucella* species, i.e., *Brucella abortus*, *B. melitensis*, and *B. suis*. Bacterial colonies from plates were blotted onto nitrocellulose disks, lysed by immersion in chloroform, and reacted with BRU 38, a rat monoclonal antibody with specificity for the O side chain of *B. abortus*. Reaction with anti-rat immunoglobulin G conjugated to horseradish peroxidase and development in 4-chloro-1-naphthol resulted in colonies of naturally occurring smooth *Brucella* species staining purple. Results could be obtained within 4 h after colonies were visible on plates and individual colonies could be detected. *Yersinia enterocolitica* serovar O:9 strains were the only other organisms tested which showed cross-reaction by using this procedure. Because of its speed, sensitivity, and specificity, this technique should be very useful for identifying smooth *Brucella* strains in diagnostic laboratories.

Brucella species are important causes of abortion and infertility in animals (18). They are also a major public health concern, because some species, namely, *Brucella abortus*, *B. melitensis*, and *B. suis*, are highly infectious for humans. These organisms are often difficult to isolate from clinical samples, and once they are isolated, the identification of *Brucella* species is based largely on a few biochemical tests and on slide agglutination reactions with test serum (6). For these reasons, positive identification of an isolate as a *Brucella* species may take several days after visible colonies have developed on the primary isolation medium. Also, the use of slide agglutination reactions may yield false-positive reactions due to cross-reacting organisms. Thus, to expedite the accurate identification of freshly isolated *B. abortus* strains, we have developed a colony blot enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody, BRU 38, with specificity for the O side chain of the lipopolysaccharides (LPS) of *B. abortus* and *B. melitensis* (15), as the primary antibody. This technique is similar to others which have been described for the detection of other bacteria and bacterial products (8, 10, 11, 16). In addition to providing a fast, specific, and sensitive means for identifying *B. abortus* isolates, the colony blot ELISA described in this report also appears to be useful for identifying isolates of *B. melitensis* and *B. suis*.

MATERIALS AND METHODS

Bacterial strains. The origins and sources of the strains used in this study are given in Tables 1 and 2. Working cultures were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants at 37°C under a 5% CO_2 atmosphere for 48 h and stored at 4°C with monthly transfer. Stock cultures were washed from slants and stored at −70°C in skim milk. The colony type of the *Brucella* strains used in this study was determined by the ability to autoagglutinate in 0.1% acriflavine and the ability of bacterial colonies to take up crystal violet (6).

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Colony blot ELISA. Bacterial strains to be tested were grown for 48 to 72 h at 37°C on Columbia agar plates supplemented with 10% defibrinated bovine or ovine blood under an atmosphere containing 5% CO_2. Nitrocellulose disks (diameter, 82 mm; Micron Separation, Inc., Honeoye Falls, N.Y.) were placed onto the plates and allowed to adhere to the surface. Disks were removed, and the adherent bacterial cells were lysed by immersing each disk in a glass petri dish containing 20 to 25 ml of chloroform for 10 min. Following lysis, the disks were allowed to dry for 15 min at room temperature and then placed into glass petri dishes containing 25 ml of a blocking-digestion buffer consisting of 0.15 M NaCl, 0.02 M Tris, 0.005 M $MgCl_2$, pH 7.5, which was further supplemented with 2 μg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml, 80 μg of lysozyme (Sigma) per ml, and 3% bovine serum albumin (Sigma). Disks were incubated in the blocking-digestion buffer at room temperature on a rotary shaker at 80 to 100 rpm for 45 min and then washed by serial passage through five glass petri dishes, each containing 25 ml of 0.5 M NaCl, 0.02 M Tris, pH 7.5 (TBS) supplemented with 0.05% Tween 20. Each disk was then placed into a glass petri dish containing a 1:10 dilution of the monoclonal antibody BRU 38 in TBS and incubated for 1 h on a rotary shaker as described above. BRU 38 is a rat immunoglobulin G monoclonal antibody produced by a hybridoma obtained from the fusion of SP2/0 myeloma cells with B cells derived from the spleens of rats immunized with *B. abortus* 19 cells (15). BRU 38 reacts with the O side chain of the *Brucella* LPS complex (15). After incubation with BRU 38, the disks were washed in TBS containing 0.05% Tween 20 as above, and each disk was placed into 15 ml of a 1:800 dilution in TBS of goat anti-rat immunoglobulin G conjugated with horseradish peroxidase, incubated on a rotary shaker as described above for 1 h, washed again in TBS containing 0.05% Tween 20, and then developed in a solution containing 60 mg of 4-chloro-1-naphthol, 10 ml of methanol, 0.6 ml of H_2O_2, and 100 ml of TBS. The reaction was stopped by immersing the disks in a large volume of distilled H_2O.

Detection of *B. abortus* colonies in a mixed culture. *B. abortus* 19 and clinical isolates of *Escherichia coli* and

TABLE 1. Reactions of *Brucella* strains in the colony blot ELISA with BRU 38

Species and strain	Colony type[a]	Origin	Reaction with BRU 38
B. abortus			
Laboratory strains			
2308	Smooth	Bovine	+
RB51	Nonsmooth	Bovine	−
19	Smooth	Bovine	+
45/20	Intermediate[b]	Bovine	+
Field strains (biovar 1)			
NVSL 7-697[c]	Smooth	Bovine	+
NVSL 7-702[c]	Smooth	Bovine	+
NVSL 7-718[c]	Smooth	Bovine	+
NVSL 7-696[c]	Smooth	Bovine	+
Field strains (biovar 2)			
NVSL 7-487[c]	Smooth	Bovine	+
NVSL 7-488[c]	Smooth	Bovine	+
NVSL 7-313[c]	Smooth	Bovine	+
NVSL 7-320[c]	Smooth	Bovine	+
Field strains (biovar 4)			
NVSL 7-625[c]	Smooth	Bovine	+
NVSL 7-534[c]	Smooth	Bovine	+
NVSL 7-619[c]	Smooth	Bovine	+
NVSL 7-627[c]	Smooth	Bovine	+
Field strains (untyped)			
W5[d]	Smooth	Bovine	+
W9[d]	Smooth	Bovine	+
W14[d]	Smooth	Bovine	+
B. melitensis			
Laboratory strains			
Rev1	Smooth	Caprine	+
B115	Nonsmooth	Caprine	−
Field strain (biovar 1) NVSL 7-93[c]	Smooth	Human	+
Field strain (biovar 2) NVSL 6-404[c]	Smooth	Human	+
B. suis			
Field strain (biovar 1) NVSL 7-223[c]	Smooth	Porcine	+
Field strain (biovar 3) NVSL 7-699[c]	Smooth	Porcine	+
B. canis			
Laboratory strain RM 666[e]	Nonsmooth	Canine	−
Field strains			
Mex 38[e]	Nonsmooth	Canine	−
Mex 11[e]	Nonsmooth	Canine	−
Mex 49[e]	Nonsmooth	Canine	−
Salerno[e]	Nonsmooth	Canine	−
41[e]	Nonsmooth	Canine	−
4236[e]	Nonsmooth	Canine	−
NVSL 7-221[c]	Nonsmooth	Canine	−
B. ovis			
Laboratory strain Wisconsin	Nonsmooth	Ovine	−
Field strain NVSL 7-75[c]	Nonsmooth	Ovine	−

[a] Colony type was determined as described in Materials and Methods.
[b] Although strain 45/20 gave positive acriflavine agglutination and crystal violet uptake reactions, it was noted in this and in a previous study (15) that strain 45/20 expresses variable amounts of O side chain depending upon culture conditions.
[c] Obtained from D. Ewalt, National Veterinary Services Laboratory, Ames, Iowa.
[d] Obtained from P. Nicoletti, College of Veterinary Medicine, University of Florida, Gainesville.
[e] Obtained from L. Carmichael, New York State College of Veterinary Medicine, Cornell University, Ithaca.

TABLE 2. Reactions in the colony blot ELISA with BRU 38 of bacteria which have previously been reported to cross-react with *Brucella* spp. in other serologic tests

Species and strain	Origin	Reaction with BRU 38
Yersinia enterocolitica		
Serovar O:9		
NCTC 11147[a]	Human	+
W191[b]	Human	+
E209/86[b]	Human	+
E223/86[b]	Human	+
E237/86[b]	Human	+
E238/86[b]	Human	+
E241/86[b]	Human	+
E248/86[b]	Human	+
E254/86[b]	Human	+
E546/86[b]	Human	+
IP383[b]	Human	+
409/36186[c]	Human	+
479/36186[c]	Human	+
518/36186[c]	Human	+
524/36186[c]	Human	+
Untyped		
1288[d]	Hamster	−
1289[d]	Porcine	−
1290[d]	Porcine	−
1291[d]	Hamster	−
1292[d]	Hamster	−
1303[d]	Hamster	−
H1[d]	Human	−
Escherichia coli serovar O:157 ATCC 35150[e]	Human	−
Salmonella urbana serovar O:30 ATCC 9261[e]	Human	−
Vibrio cholerae biovar Inaba ATCC 11628[e]	Human	−
Campylobacter fetus subsp. *fetus* ATCC 27374[f]	Ovine	−
Campylobacter fetus subsp. *venerealis* ATCC 19438[f]	Bovine	−
Pasteurella sp. strain R1894[g]	Porcine	−

[a] Obtained from the National Collection of Type Cultures, London, United Kingdom.
[b] Obtained from G. Wauters, Université Catholique de Louvain, Brussels, Belgium.
[c] Obtained from S. Aleksic, Freie und Hansestadt, Hamburg, Federal Republic of Germany.
[d] Obtained from C. Gebhart, College of Veterinary Medicine, University of Minnesota, St. Paul.
[e] Obtained from the American Type Culture Collection, Rockville, Md.
[f] Obtained from L. M. Thompson, Biology Department, Virginia Polytechnic Institute and State University, Blacksburg.
[g] Obtained from D. Helms, Virginia Department of Agriculture and Consumer Services, Ivor. This strain agglutinated in both porcine and bovine *B. abortus*-positive test sera. Although it has been tentatively identified as a member of the genus *Pasteurella* based on its biochemical characteristics, it does not fit any of the recognized species in this genus.

Streptococcus faecalis obtained from the Diagnostic Bacteriology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine were grown on Trypticase soy agar plates for 48 h at 37°C under 5% CO_2. Growth from each culture was suspended in sterile phosphate-buffered saline, the optical density of each suspension was adjusted to 0.5 at

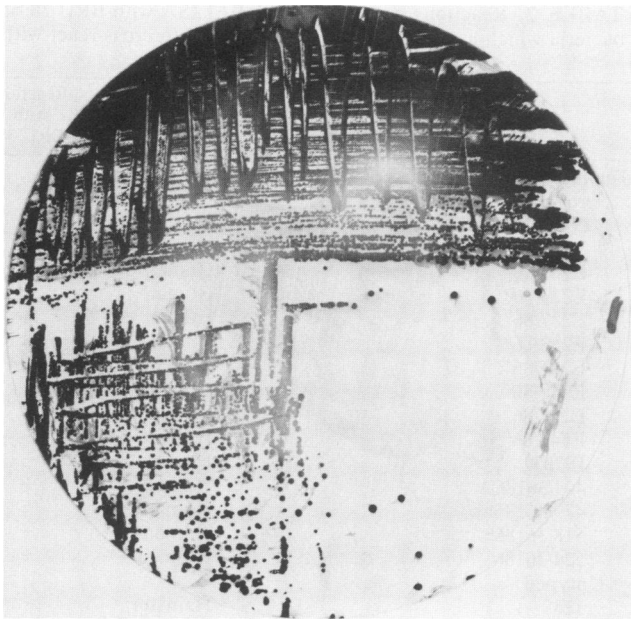

FIG. 1. Reaction of a 48-h culture of *B. abortus* NVSL 7-697 blotted onto nitrocellulose and subjected to the colony blot ELISA with BRU 38.

525 nm, and 10-fold dilutions were made in phosphate-buffered saline. A 0.3-ml sample of the 10^{-3} dilution of the *B. abortus* 19 suspension was mixed with 0.3 ml of the 10^{-4} dilution of the *E. coli* suspension and 0.3 ml of the 10^{-4} dilution of the *S. faecalis* suspension. Each of these dilutions contained approximately 10^6 CFU/ml as determined by standard plate count procedures. A 10-μl sample of the mixture was placed onto each of three Columbia blood agar plates and streaked with a sterile loop; 10 μl of each of the dilutions used in the mixture were also streaked individually onto Columbia blood agar plates. Plates were incubated for 48 h at 37°C with 5% CO_2, and the growth from the plates was used in the colony blot ELISA described above.

RESULTS

For all of the bacterial strains tested, colonies were easily blotted onto nitrocellulose disks by using the described procedure. The disks wetted quickly and sealed tightly to the surface of the medium. When the disks were removed, the majority of the growth from the plate was stuck to the nitrocellulose membrane, forming a mirror image of the growth pattern on the plate. Colonies having antigens recognized by BRU 38 showed up as purple areas on the white background of the nitrocellulose disks (Fig. 1). *B. abortus* 19 colonies could also be distinguished in mixed culture with *E. coli* and *S. faecalis* (Fig. 2).

All of the naturally occurring smooth *Brucella* strains representing *B. abortus*, *B. melitensis*, and *B. suis* tested in this assay yielded positive results. Conversely, all of the *B. canis* and *B. ovis* strains tested, which occur naturally in the nonsmooth phase, gave negative results. Stable laboratory-derived rough mutants of *B. abortus* (RB51) and *B. melitensis* (B115) were also negative in this assay. Of the other organisms tested, the only ones that gave positive reactions in this assay were serovar O:9 strains of *Yersinia enterocolitica* (Table 2).

DISCUSSION

Diagnosis of brucella infections is complicated by the fact that these organisms are often difficult to isolate from clinical samples and once isolated there are very few biochemical tests available to confirm the identification of the isolate. Agglutination in positive serum is sometimes used as part of the regimen for identifying *B. abortus*; however, this approach can cause problems because there are several other gram-negative bacteria which can show serologic cross-reaction with *B. abortus* (5, 7, 9, 12–14, 17).

The technique described in this report makes use of a monoclonal antibody, BRU 38, which is directed against the O side chain (perosamine polymer; 4) of the LPS of *B. abortus* (15). Because *B. melitensis* and *B. suis* also contain perosamine as a constituent of their LPS O side chains (2, 6), these organisms are also recognized by BRU 38. Hence, the usefulness of the assay described here is not limited to detecting *B. abortus* on primary isolation. This should make it especially useful in the diagnosis of human brucella infections. BRU 38 also recognizes *Y. enterocolitica* serovar O:9 strains, which is not surprising, since these organisms contain perosamine as the O side chain of their LPS (3). However, this should pose no great problem in diagnostic laboratories, as *B. abortus* is easily distinguished from *Y. enterocolitica* on the basis of simple biochemical tests, such as reaction on a TSI slant (1, 6).

The sensitivity, specificity, and speed of this test are three of its major advantages over the currently used procedures for the preliminary identification of *B. abortus*, *B. melitensis*, or *B. suis* in the diagnostic laboratory. Individual *B. abortus* colonies which are barely visible on a plate can be clearly visualized on nitrocellulose by using this procedure. Thus, a primary culture can be identified earlier than usual. In addition, the primary culture does not have to be pure. We were able to detect *B. abortus* colonies grown on a plate in an equal mixture with clinical isolates of *E. coli* and *S.*

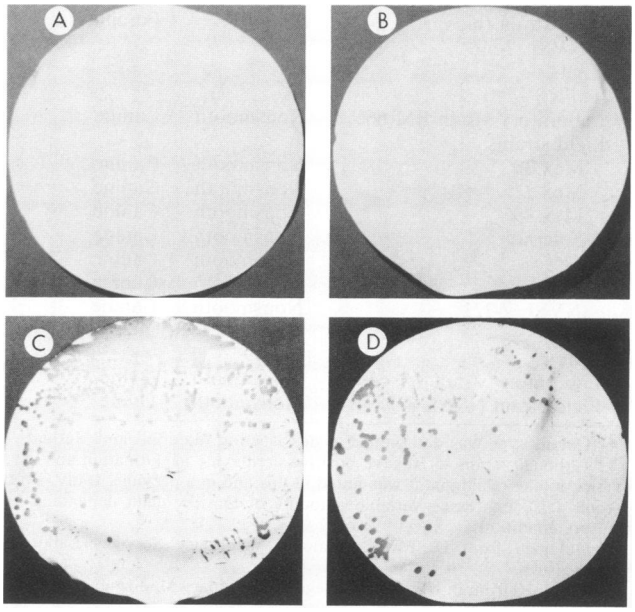

FIG. 2. Reaction of 48-h cultures of *E. coli* (A), *S. faecalis* (B), *B. abortus* 19 (C), and a mixed culture of these organisms (D) blotted onto nitrocellulose and subjected to the colony blot ELISA with BRU 38.

faecalis (Fig. 2). However, selective media should still be used, as some tissue contaminants appear to be able to prevent the growth of *B. abortus* on a nonselective medium (R. M. Roop, unpublished data). Finally, as little as 1 h seems to be sufficient for incubation of blotted disks with both BRU 38 and the secondary antibody when they are used at the dilutions described. With time allowed for washing between incubations and for the lysis and blocking steps, tentative results indicating the presence of *Brucella* spp. can be obtained as soon as 4 h after colonies are visible on a primary isolation medium. If sterile (autoclaved) nitrocellulose disks are used, plates can be reincubated and a colony which yielded a positive reaction can be removed and used to confirm the identification by routine biochemical procedures and for shipment to a reference laboratory for biotyping.

Because of its speed, specificity, and sensitivity, the procedure described in this report should be very useful in both human and veterinary diagnostic laboratories for the identification of *B. abortus*, *B. melitensis*, and *B. suis*. It will provide small laboratories with a dependable method for rapidly identifying these virulent organisms without having to rely on reference laboratories. Work is under way in our laboratory to expand this technique to *B. canis* and *B. ovis* isolates through the use of other recently developed monoclonal antibodies.

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