JOURNAL OF CLINICAL MICROBIOLOGY, July 1997, p. 1794–1799 Vol. 35, No. 7
0095-1137/97/$04.00+0

Characterization of a Specific *Mycobacterium paratuberculosis* Recombinant Clone Expressing 35,000-Molecular-Weight Antigen and Reactivity with Sera from Animals with Clinical and Subclinical Johne's Disease

FOUAD A. K. EL-ZAATARI,[1,2]* SALEH A. NASER,[3] AND DAVID Y. GRAHAM[1,2,4]

Inflammatory Bowel Disease Laboratory, Veterans Affairs Medical Center,[1] and Department of Medicine[2] and Division of Molecular Virology,[4] Baylor College of Medicine, Houston, Texas, and Department of Molecular Biology and Microbiology, University of Central Florida, Orlando, Florida[3]

Received 14 October 1996/Returned for modification 30 January 1997/Accepted 24 March 1997

Johne's disease is a chronic enteritis of ruminants associated with enormous worldwide economic losses for the dairy cow- and goat-rearing industries. Management limitations and eradication programs for this disease have been hampered by the lack of a simple and specific diagnostic test for the detection of subclinical cases. We used a recombinant clone expressing a 35,000-molecular-weight *Mycobacterium paratuberculosis* antigen (p35 antigen) from a previously constructed expression library of *M. paratuberculosis* in *Escherichia coli*. The DNA fragment encoding the p35 gene hybridized only to DNA from *Mycobacterium avium* complex, but not to DNAs from other mycobacteria and nonmycobacterial organisms. The seroreactivity of p35 was evaluated by immunoblotting against 57 reference serum samples obtained from infected and uninfected animals. p35 was recognized by sera from 100% of animals with advanced Johne's disease (clinical stage) (12 cattle, 2 goats, and 2 sheep) and by sera from 75% of 20 cattle with early infection (subclinical stage). None of the sera from 15 *M. paratuberculosis*-free cows, 3 *Mycobacterium bovis* BCG-infected tuberculous cattle, or 3 cows artificially inoculated with multiple doses of viable *M. paratuberculosis* reacted with p35. The overall sensitivity, specificity, positive predictive value, and negative predictive value were 86, 100, 100, and 75%, respectively. The accuracy of p35 immunoblotting was superior to those of commercially available diagnostic tests for Johne's disease. These results suggest that the p35 recombinant protein has potential for use in the serodiagnosis of animals with Johne's disease at all stages of infection. The DNA fragment encoding p35 may also serve as a probe for identification of *M. avium* complex infection.

Paratuberculosis (Johne's disease) is a chronic inflammatory bowel syndrome in domestic and wild ruminants characterized by granulomatous enteritis, diarrhea, and emaciation (7). The etiologic agent, *Mycobacterium paratuberculosis*, is an extremely slowly growing mycobactin-dependent organism (7). Although, generally diagnosed in ruminants, *M. paratuberculosis* has also been isolated from subhuman primates suffering from chronic ileitis and has recently been implicated in Crohn's disease and sarcoidosis in humans (7, 8, 14, 19, 20, 23, 27, 37).

Johne's disease occurs worldwide and throughout the United States (7). It has been estimated that 5 to 20% of cattle in the United States are infected, and the economic losses are enormous. Dairy farmers with infected herds may lose $75 to $100 per adult head, for estimated total losses in the United States of more than $1.5 billion annually (7, 32). On the basis of prevalence data, the economic significance of Johne's disease to the New England states, Wisconsin, and Florida were in excess of $15 million, $54 million, and $9 million annually, respectively (2, 5, 7). At present there is no specific therapy or effective control or vaccination programs against Johne's disease. Although good management to control this disease will lead to a reduced incidence, eradication is dependent on detection and culling of infected animals as early as possible. Eradication programs and the management limitations for this disease have been hampered by the lack of simple and specific diagnostic tests for detecting the disease in subclinically infected (infected but symptom-free) animals (1, 7, 10, 24, 29–31). Recently, gene probes and PCR assays for the detection of *M. paratuberculosis* in feces have been developed (35, 38). However, these nucleic acid-based techniques are reported to require specialized equipment, to be expensive, and to be less sensitive (sensitivities, 3 to 23%) than conventional fecal culture (24, 35).

Research efforts have also been directed toward the development and application of new and improved species-specific serologic tests for the identification of subclinical paratuberculosis. An array of these tests has been developed for detecting antibodies in the sera of infected animals. Although serologic tests are rapid and easy to perform, the results have generally been unsatisfactory (1, 2, 7, 10, 28, 31, 33). Most tests depend on the use of either partly purified *M. paratuberculosis* or whole cytoplasm of *Mycobacterium avium* 2 (previously known as *M. paratuberculosis* 18) (24, 36). The specificities of these tests have been poor due to antigenic cross-reactivity between *M. paratuberculosis* and other mycobacterial species and nonmycobacterial organisms (2, 7, 10, 17, 21, 28, 31, 33). To date, the most accurate diagnostic methods for the detection of clinical cases of infection remain microscopy of Ziehl-Neelsen-stained fecal smears and fecal culture. However, staining identifies only 25 to 35% of fecal culture-positive cattle and culture requires an incubation period of 8 to 12 weeks. Furthermore, fecal culture is of limited value because the minimum detection limit cannot detect all subclinical infections (4, 7). Although current serologic tests and fecal culture are useful

* Corresponding author. Mailing address: VA Medical Center (111D), 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 795-0232. Fax: (713) 790-1040. E-mail: fouade@bcm.tmc.edu.

TABLE 1. Bacterial strains tested by dot blot hybridization

Strain	Source[a]
Acinetobacter calcoaceticus	VAMC
Campylobacter jejuni	ATCC 1287
Citrobacter diversus	VAMC
Escherichia coli[b]	Invitrogen, Inc.
Enterococcus faecalis	VAMC
Helicobacter pylori RD26	VAMC
Klebsiella pneumoniae	ATCC 13883
Morganella morganii	VAMC
Mycobacterium avium MAIS 2	NADC
Mycobacterium avium serovar 7	CSU
Mycobacterium avium serovar 9	CSU
Mycobacterium avium serovar 19	CSU
Mycobacterium avium wood pigeon VI-72	CSU
Mycobacterium fortuitum	ATCC 6841
Mycobacterium kansasii	ATCC 12478
Mycobacterium paratuberculosis Linda	ATCC 43015
Mycobacterium paratuberculosis Ben	ATCC 43544
Mycobacterium paratuberculosis C286	CSU
Mycobacterium paratuberculosis	ATCC 19698
Mycobacterium tuberculosis	ATCC 25177
Mycobacterium smegmatis	ATCC 27199
Mycobacterium chelonae	VAMC
Mycobacterium phlei	VAMC
Nocardia asteroides	VAMC
Proteus mirabilis	ATCC 7002
Pseudomonas aeruginosa	VAMC
Serratia marcescens	VAMC
Shigella flexneri	VAMC
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228
Viridans group streptococci	VAMC
Vibrio parahaemolyticus	VAMC
Vibrio cholerae	VAMC

[a] VAMC, Veterans Affairs Medical Center, Houston, Tex.; ATCC, American Type Culture Collection, Rockville, Md.; NADC, Mycobacteriosis Unit, National Animal Disease Center, Ames, Iowa; CSU, Department of Microbiology, Colorado State University, Fort Collins.

[b] INVαf′, a derivative of DH1 strain (Invitrogen Corp.).

in detecting cattle with clinical paratuberculosis, the application of these procedures in identifying cattle in early stages of infection or in subclinical stages has proven to be of limited value (28, 33).

Development of sensitive and specific serologic tests for the rapid identification of infected animals requires identification of a specific protein antigen(s) or epitope(s) for *M. paratuberculosis*. We previously constructed an *M. paratuberculosis* genomic library in an expression vector to aid in isolating a speces-specific antigen(s) and/or epitope(s) that could be used for the development of a specific serologic test for Johne's disease and, perhaps when its link is proven, for Crohn's disease (12, 15). We now describe the isolation and characterization of a recombinant clone expressing a 35,000-molecular-weight antigen (35K antigen) of *M. paratuberculosis* (p35 antigen). We also evaluated the seroreactivity of this molecule against sera obtained from reference animals with Johne's disease and from humans with Crohn's disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The reference strains and clinical isolates listed in Table 1 were used to prepare DNA. The mycobacterial strains were grown in 7H9 broth (pH 5.9) supplemented with 100 ml of Middlebrook OADC (oleic acid, albumin, dextrose, catalase) enrichment (Difco Laboratories, Detroit, Mich.) per liter, 0.1% glycerol, and 0.05% Tween 80. Mycobactin J (Allied Monitor, Inc., Fayette, Mo.) was added to the broth for culturing *M. paratuberculosis* and *M. avium* strain wood pigeon at a final concentration of 2 mg/liter as described previously (14). *Escherichia coli* (INVαf′, a derivative of strain DH1; Invitrogen Corp., San Diego, Calif.) and its recombinant variants were grown in Luria-Bertani (LB) medium containing 200 μg of ampicillin per ml, as suggested by the manufacturer. The clinical isolates were kindly provided by Jill Clarridge (Clinical Microbiology Laboratory, Veterans Affairs Medical Center, Houston, Tex.) and were isolated and identified by standard microbiologic procedures (25). Stock cultures were each stored in medium supplemented with 25% glycerol at −80°C.

Preparation of DNA. Chromosomal DNAs from the reference strains and clinical isolates were prepared from freshly harvested bacterial cells. After being washed twice with sterile saline (150 mM NaCl), each bacterial pellet was suspended in 2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing lysozyme (egg white, grade 1; Sigma Chemical, Co., St. Louis, Mo.) at 3.5 mg/ml. After incubation for 30 min at 37°C, proteinase K (0.1 mg/ml) and sodium dodecyl sulfate (SDS; 1% [wt/vol]) were added and the mixture was incubated for additional 2 h at 55°C. The lysate was treated with RNase A (GIBCO BRL, Gaithersburg, Md.) at a final concentration of 100 μg/ml and incubated further for 60 min at 37°C. The DNA was extracted twice with an equal volume of a mixture of phenol-chloroform–isoamyl alcohol (24:1 [vol/vol]). After another extraction with chloroform-isoamyl alcohol, the DNA was ethanol precipitated, spooled out, and dissolved in TE buffer. The DNA concentration was determined spectrophotometrically at 260 nm.

Genomic library and DNA manipulations. The construction and screening of our *M. paratuberculosis* expression library have been described previously (12, 15). The DNA from *M. paratuberculosis* Linda (ATCC 43015), an isolate from a patient with Crohn's disease, and pcDNAII expression phagemid vector (Invitrogen Corp.) were used to construct this library. Twenty-four recombinant clones containing *Bam*HI inserts of between 1.6 and 4.2 kb were also purified and identified by hyperimmune rabbit anti-*M. paratuberculosis* screening serum (15). One recombinant clone containing a 3.2-kb fragment of *M. paratuberculosis*, designated clone pMptb #40, was selected for protein analysis. Plasmid DNA was prepared by the alkaline lysis method (26). Restriction endonuclease digestions were performed as recommended by the manufacturer (GIBCO BRL Life Technologies, Inc., Grand Island, N.Y.).

In vitro transcription-translation. One microgram of *Xho*I-linearized recombinant phagemid (from clone pMptb #40) was used as a template to perform in vitro transcription with the Riboprobe Gemini II Core system with Sp6 RNA polymerase, as recommended by the manufacturer (Promega Corp., Madison, Wis.). A small aliquot of the synthesized mRNA was visualized on a 1% neutral agarose gel, and the transcribed sample was treated with RNase-free DNase I (1 U/μg; Promega) at 37°C for 15 min. mRNA templates from the transcription reaction were used for the in vitro translation reaction by using the tRNA^{nscend} Non-Radioactive Translation Detection System as directed by the manufacturer (Promega). Four microliters of the mRNA from the transcription reaction was mixed with rabbit reticulocyte lysate (Promega), an amino acid mixture (1 mM), RNasin RNase inhibitor (40 U), and 1 μl of biotin-labeled lysine tRNA^{nscend}, and the mixture was incubated at 30°C for 2 h. Luciferase mRNA provided by the manufacturer of the translation system was used as the positive control, as suggested by the manufacturer (Promega). The biotinylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and blotting on Immobilon-P transfer membranes (polyvinylidene difluoride membranes; Millipore Intertech, Bedford, Mass.) (13, 15). Luciferase protein and p35 were visualized on blot strips by binding to streptavidin-alkaline phosphatase, followed by color detection with a substrate that contains nitroblue tetrazolium (Boehringer Mannheim, Indianapolis, Ind.) and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim), as described by the manufacturer. The reactivity of p35 was also visualized with an immunoblot strip that was incubated with adsorbed rabbit anti-*M. paratuberculosis* serum as described previously (13, 15).

Serum samples. Rabbit hyperimmune anti-*M. paratuberculosis* serum was produced in our laboratory by repeated subcutaneous immunization with *M. paratuberculosis* sonicate as described previously (15). Briefly, the bacilli were grown, washed, suspended in saline (at 0.5 g of bacilli per 1 ml), and sonicated with an ultrasonic cell disrupter (model 250; Sonic & Materials, Inc., Danbury, Conn.) in an ice-water bath until they were translucent. A dose of clarified sonicate containing 0.5 mg of mycobacterial proteins was emulsified in incomplete adjuvant (1:1 ratio [vol:vol]; Sigma Chemical Co.) and administered intramuscularly to a New Zealand White rabbit. At the end of the 4th week and weekly thereafter, the animal was boosted intravenously with 100 μg of mycobacterial proteins in 100 μl of saline. After the 8th week of immunization the rabbit was anesthetized and exanguinated, and the antiserum was stored at −70°C. A total of 57 animal serum samples obtained from the Mycobacteriosis Unit, National Animal Disease Center, Ames, Iowa; School of Veterinary Medicine, University of Wisconsin, Madison; and Allied Monitor, Inc., were used. Among these were samples from 2 sheep, 2 goats, 12 cows with clinical Johne's disease, 20 cows with subclinical naturally acquired Johne's disease, and 3 cows inoculated with *M. paratuberculosis* 2 cows [cows 861 and 862] were artificially inoculated with *M. paratuberculosis* 19698 at the source of origin [National Animal Disease Center], 1 cow was inoculated with *M. paratuberculosis* 3737 by Chiodini and Davis [6]), and 15 healthy cows without detectable *M. paratuberculosis* infection. Additionally, three serum samples from cows infected with *Mycobacterium bovis* were used as controls. Human serum samples from three patients with Crohn's disease and four patients with tuberculosis obtained from patients attending a Veterans

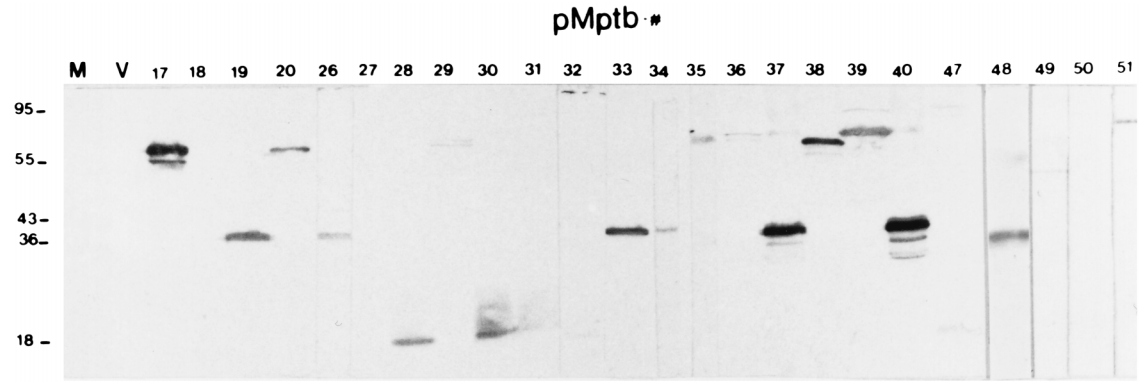

FIG. 1. SDS-immunoblot reactivities of purified *M. paratuberculosis* recombinant clones with rabbit anti-*M. paratuberculosis* serum. Lysates of recombinant *E. coli* expressing *M. paratuberculosis* antigens were fractionated on SDS-immunoblot strips. The reactivities of the recombinant antigens on these strips were evaluated by incubating them with rabbit anti-*M. paratuberculosis* serum. The numbers on the top (pMptb numbers) designate the number of each positive clone that was purified by rabbit antibody screening. Lane V, fractionated proteins of *E. coli* containing the vector pcDNAII only as a negative control; lane M, molecular weight markers. The numbers to the left are molecular weights (in thousands).

Affairs hospital and from six patients with leprosy obtained from P. Brennan, Colorado State University, Fort Collins, were also tested. In addition, rabbit anti-*M. avium* serovar 9 serum and rabbit anti-*Mycobacterium intracellulare* serovar 12 serum obtained from Mycobacteriology Laboratory Respiratory Disease Branch, Centers for Disease Control and Prevention, Atlanta, Ga., were also used. All sera were adsorbed by a mixture of nonrecombinant *E. coli* (INVαf' a derivative of strain DH1 from Invitrogen Corp.) sonicate suspended in 2 ml of phosphate-buffered saline (0.01 M $Na_2HPO_4 \cdot 7H_2O/NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl [pH 7.0]) and a boiled pellet, each prepared from 100 ml of overnight LB broth cultures containing 100 μg of ampicillin per ml. Adsorption was at a dilution of 1:30 (vol/vol; serum and sonicate-pellet mixture) for 16 h at 4°C. The efficiency of adsorption was evaluated by immunoblotting against *M. paratuberculosis* sonicate and *E. coli* lysate proteins as we described previously for the rabbit antiserum (12, 13, 15). The adsorbed serum was stored at −70°C in aliquots until it was used.

Immunoblotting and dot blot hybridization. SDS-immunoblotting (with 12% [wt/vol] acrylamide) was performed by using Immobilon-P transfer membranes (Millipore Intertech) as described previously (13, 15). Immunoblot strips containing fractionated proteins were tested against individual adsorbed sera as described previously (13, 15).

Plasmid from immunoreactive clone pMptb #40 was digested, and the *M. paratuberculosis Bam*HI-restricted fragment of 3.2 kb was electroeluted from the agarose gel with a DEAE-cellulose membrane (NA-45; Schleicher & Schuell, Keene, N.H.) as described previously (12, 15). This DNA fragment was labeled by the hexanucleotide priming technique with digoxigenin-11-dUTP by using the Genius labeling kit following the manufacturer's instructions (Boehringer Mannheim). Dot blot hybridization with the digoxigenin-labeled probe was performed by following the manufacturer's instructions (Boehringer Mannheim). Briefly, 3 μg of each denatured chromosomal DNA (with 0.2 M NaOH containing 20 mM EDTA at 100°C for 10 min) was placed onto nylon membranes (Hybond-N^+; Amersham, Arlington Heights, Ill.) by using the minifold system (Bio-Rad, Richmond, Calif.). Hybridization with digoxigenin-labeled probes and detection were performed by using the Genius kit as directed by the manufacturer (Boehringer Mannheim).

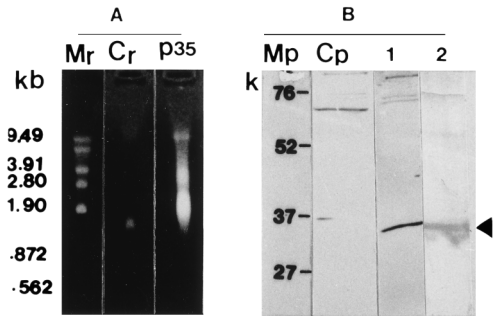

FIG. 2. Identification and expression of p35 by in vitro transcription-translation. A linearized recombinant phagemid (from clone pMptb #40) was used in the in vitro transcription-translation reaction with rabbit reticulocyte lysate by using biotin-labeled lysine tRNA. The biotinylated proteins were analyzed by SDS-Western blotting (SDS-immunoblotting) and by binding to streptavidin-alkaline phosphatase or immunoblotting (with hyperimmune rabbit-anti *M. paratuberculosis* serum), followed by color detection with the appropriate substrate as described in the text. (A) Ethidium bromide-stained agarose gel electrophoresis of mRNA templates of the lusiferase gene as a positive control (Cr) and the p35-encoding gene. (B) SDS-immunoblotting analysis of the translated lusiferase gene product as a positive control (Cp) and the p35 gene product developed by a streptavidin-alkaline phosphatase protocol (lane 1) and by rabbit anti-*M. paratuberculosis* sera (lane 2). M_r, RNA molecular weight marker (the numbers to the left are molecular masses [in kilobases]); Mp, molecular weights (in thousands) of standard proteins. The arrowhead indicates the location of p35 antigen.

RESULTS

We used a previously constructed *M. paratuberculosis* genomic expression library and 24 recombinant clones purified by antibody screening (12, 15). These clones were analyzed and tested against rabbit anti-*M. paratuberculosis* serum by the SDS-immunoblotting technique (15). The reacting bands representing the expressed products of these clones are shown in Fig. 1. The molecular weights of these products varied from 19,000 to 80,000. One clone (clone pMptb #40) expressing a 35K antigen (p35 antigen) reacted very strongly with combined sera from cattle, goats, and sheep with Johne's disease but not with combined sera from *M. bovis* BCG-infected tuberculous cows. Hence, the characterization and the seroreactivity of this clone were evaluated.

In vitro transcription-translation. The antigenicity and the molecular weight of the p35 antigen were verified by analyzing the in vitro transcription-translation product of the recombinant phagemid of the pMptb #40 clone (Fig. 2). Migration of protein products indicated an apparent molecular weight of about 62,000 for luciferase protein (the positive control) and for the 35K product (p35) (Fig. 2B, lanes Cp and 1, respec-

TABLE 2. Summary of overall reactivity of p35 against serum samples from animals[a]

Diagnosis[b]	No. of positive samples/ total no. tested
Clinical	16/16
Subclinical	15/20
M. bovis infected	0/3
Inoculated	0/3
Healthy	0/15

[a] None of the animals was vaccinated.
[b] Definitive results based on all tests conducted at the source of samples.

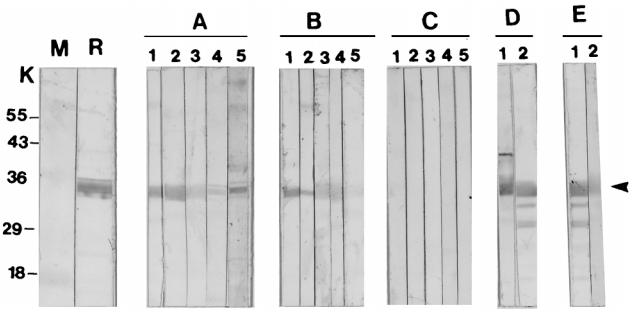

FIG. 3. Representative immunoblot analysis of *M. paratuberculosis* p35 antigen. Lysates of recombinant *E. coli* expressing *M. paratuberculosis* p35 antigen were fractionated on SDS-immunoblot strips. The reactivity of *M. paratuberculosis* recombinant p35 antigen on these strips was evaluated with sera from cows with Johne's disease, clinical stage (A); cows with Johne's disease, subclinical stage (B); *M. paratuberculosis*-free cows (C); sheep with Johne's disease, clinical stage (D); and goats with Johne's disease, clinical stage (E). The numbers on top represent each serum sample. Lane R, a strip incubated with rabbit anti-*M. paratuberculosis* serum; lane M, molecular weight markers (the numbers to the left are molecular weights [in thousands]). The arrowhead indicates the location of the p35 antigen.

tively). The antigenicity of the expressed p35 antigen was verified by its reactivity with the rabbit anti-*M. paratuberculosis* serum (Fig. 2B, lane 2).

Seroreactivity of p35 antigen. The results of p35 seroreactivity are summarized in Table 2. The infection status of all animals was confirmed by isolation of the organism from fecal and tissue samples at the source of samples. p35 was recognized by sera from all 16 (100%) reference animals with advanced Johne's disease (clinical stage) (12 cattle, 2 goats, and 2 sheep) and 15 of 20 (75%) reference cattle with early infection (subclinical stage). p35 was not recognized by sera from three BCG-infected cows (tuberculous serology positive) or three cows experimentally inoculated with viable *M. paratuberculosis* organisms. In addition, p35 did not react with sera from 15 *Mycobacterium paratuberculosis*-free control cows. Samples were considered positive when a band (faint or strong) was observed at the expected molecular weight of 35,000, as indicated in Fig. 3. As expected when dealing with recombinant *E. coli* clones, nonspecific bands were also seen on strips incubated with some serum samples, indicating the incomplete adsorption of anti-*E. coli* antibodies in some of these samples. The overall results including the sensitivity, specificity, positive

TABLE 3. Comparative serologic analysis of paratuberculosis in cattle[a]

Animal group and no.[b]	Serologic test results					
	CF[c]	AGID[d]	Allied ELISA[e]	CSL ELISA[f]	UW ELISA[g]	p35 blots[h]
Healthy cows[i]						
134 A9	−	ND[j]	−	−	−	−
165 A9	−	ND	−	−	−	−
135 A9	−	ND	−	−	−	−
104 A9	−	ND	−	−	−	−
101 A9	−	ND	−	−	−	−
106 A9	−	ND	−	−	−	−
137 A9	−	ND	−	−	+	−
108 A9	−ve	ND	−	−	−	−
138 A9	−ve	ND	−	−	−	−
102 A9	−ve	ND	−	−	−	−
No. of positive samples/total no. tested (sensitivity [%])	0/10 (100)	0/10 (100)	0/10 (100)	9/10 (90)	0/10 (100)	
Cow with subclinical infection[i]						
63 C8	−	−	−	−	+	+
16 A9	+	+	+	+	+	+
211 D9	−	+	−	+	+	+
208 D9	+	+	+	+	+	+
301 D9	+	+	+	+	+	+
329 D9	−	−	−	−	−	+
350 D9	−	−	−	−	−	+
159 F9	−	−	+	+	+	+
147 F9	−	−	−	−	−	−
124 F9	−	−	+	−	+	−
177 F9	−	−	+	−	−	+
352 D9	−	−	+	−	−	+
333 F9	−	−	+	+	−	−
327 F9	+	−	−	−	−	−
8 C8	−	−	−	−	−	+
No. of positive samples/total no. tested (sensitivity [%])	4/15 (27)	4/15 (27)	8/15 (53)	6/15 (40)	7/15 (4)	11/15 (73)

[a] None of the animals was vaccinated.
[b] Accession number of the U.S. National Repository for Paratuberculosis Specimens.
[c] CF, complement fixation test.
[d] AGID, agarose immunodiffusion test.
[e] The assay used partly purified antigen from *M. paratuberculosis* 18, which was recently reclassified as *M. avium* 18.
[f] The assay used the whole cytoplasm of *M. paratuberculosis*. CSL, Commonwealth Serum Laboratory.
[g] UW, University of Wisconsin.
[h] Recombinant clone pMptb #40 was fractionated on SDS-immunoblots.
[i] Definitive results based on all tests, including fecal and diseased tissue cultures, conducted at the source of samples.
[j] ND, not determined.

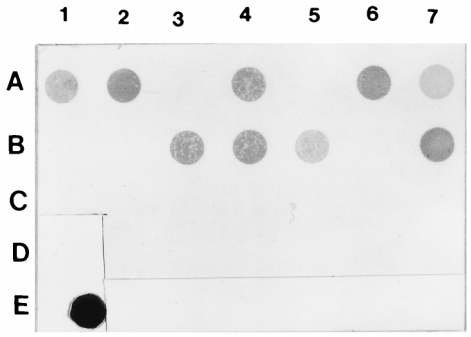

FIG. 4. Dot blot hybridization of DNA from mycobacteria and nonmycobacterial organisms with digoxigenin-labeled pMptb #40 DNA insert probe. The chromosomal DNA samples that were tested were from *M. avium* (serovar 7), *M. paratuberculosis* Linda (ATCC 43015), *M. chelonae*, *M. paratuberculosis* Ben (ATCC 43544), *M. phlei*, *M. paratuberculosis* C286, and *M. paratuberculosis* ATCC 19698; (A1 to A7, respectively); *Mycobacterium kansasii* ATCC 12478, *M. tuberculosis* ATCC 25177, *Mycobacterium avium* MAIS 2, *M. avium* serovar 9, *M. avium* serovar 19, *Mycobacterium fortuitum* ATCC 6841, and *M. avium* wood pigeon VI-72 (B1 to B7, respectively); *Morganella morganii*, *Mycobacterium smegmatis* ATCC 27199, *Staphylococcus aureus* ATCC 25923, *Nocardia asteroides*, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa*, and *Campylobacter jejuni* ATCC 1287 (C1 to C7, respectively); *Citrobacter diversus*, *Klebsiella pneumoniae* ATCC 13883, *Shigella flexneri*, *Serratia marcescens*, *Proteus mirabilis* ATCC 7002, *Enterococcus faecalis*, and *Vibrio parahaemolyticus* (D1 to D7, respectively); and pMptb #40 DNA fragment (positive control), *Vibrio cholerae*, *Acinetobacter calcoaceticus*, viridans group streptococci, *E. coli*, and *Helicobacter pylori* RD26 (E1 to E6, respectively).

predictive value, and negative predictive value for p35 were 86.1, 100, 100, and 75%, respectively.

p35 reacted weakly with sera from one of four (25%) patients with tuberculosis and two of six (33%). patients with leprosy; there was no reactivity with sera from three patients with Crohn's disease. Moreover, p35 reacted to rabbit anti-*M. intracellulare* serovar 12 serum but not to rabbit anti-*M. avium* serovar 9 serum.

Comparison of p35 seroreactivities with those of other tests for Johne's disease. Table 3 summarizes p35 immunoblot assay results and other serological test results performed with 25 reference serum samples from cattle (10 serum samples from healthy cattle and 15 serum samples from cattle with subclinical Johne's disease). The serological data of these reference samples was provided by M. Collins, U.S. National *M. paratuberculosis* Repository, University of Wisconsin. When these data were compared, the 73% sensitivity of the p35 assay exceeded the sensitivities of the other widely used tests: 53, 40, 47, 27, and 27% for the Allied enzyme-linked immunosorbent assay (ELISA; Allied Monitor, Inc.), the CSL ELISA (Commonwealth Serum Laboratory, Parkville, Australia), the UW ELISA (test performed at the University of Wisconsin), the agarose gel immunodiffusion test (Rapid John's test; Immucell Corp, Portland, Maine), and the complement fixation test (21), respectively. The specificities were comparable for all assays.

Hybridization. When the 3.2-kb *Bam*HI-digested DNA insert of the pMptb #40 clone was labeled and used as a probe, it hybridized only to DNAs from the nine isolates of the *M. avium* complex, including *M. paratuberculosis*. There was no hybridization with the DNAs from the other six *Mycobacterium* sp. isolates or with the DNAs from 16 unrelated strains, including the closely related species *Nocardia asteroides*, that were tested (Fig. 4).

DISCUSSION

In mycobacterial infection, it is not known how many antigens or epitopes are involved in the development of disease or in protective immunity. This is due to the combined humoral and cell-mediated immune responses elicited by the actively infected host (17). Like other mycobacteria, it is difficult to analyze the antigenic structure of *M. paratuberculosis* due to its extremely slow growth and cross-reactivity with other mycobacterial and nonmycobacterial species (7, 17, 21). These problems are common to the serologic diagnosis of all mycobacterial infections (17). Thus, serologic identification of mycobacterial species may be demonstrated only at the epitope level and is rarely demonstrated at the component level (3). Antigens of *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and *M. bovis* have been cloned in expression vector gt11 (34, 39), enabling the isolation of recombinant antigens that carry species-specific determinants. Detailed mapping of these determinants has been established by subsequent subcloning (16, 22). Therefore, it is conceivable that a purified antigenic component(s) with a specific epitope(s) of *M. paratuberculosis* could provide the basis for a rapid diagnostic test for Johne's disease.

With improved cultural techniques and the use of PCR-based assays, more *M. paratuberculosis* isolates have been isolated from or identified in tissues from human patients with Crohn's disease or sarcoidosis (14, 19, 23, 27, 37). The poor reactivity of p35 with human serum samples by no means rules out the possible association of *M. paratuberculosis* with these human diseases, but, rather, p35 may not be a molecule immunogenic to the human humoral immune response. However, more human serum samples need to be tested against this molecule to confirm whether p35 is a weak immunogen for humans. Recently, we described a p36 molecule from the same library that reacted strongly to serum samples from patients with mycobacterial diseases but not to serum samples from animals with Johne's disease (14). Moreover, the reactivity of the p35 antigen with rabbit anti-*M. intracellulare* serum but not with anti-*M. avium* serum indicates that the p35 antigen cross-reacts with an antigen or epitope(s) found on *M. intracellulare* that is perhaps not found on *M. avium*. It has also been suggested that contaminated food such as milk and milk products or meat and meat products from infected animals may be a source of infection with this potential pathogen for humans (11, 18). Development of accurate serologic tests for the detection of Johne's disease at all stages of infection would assist in Johne's disease eradication programs.

In this study, we used an immunoblot-based assay and showed that p35 sensitivity (73%) exceeded those of other widely used tests when they were evaluated with sera from the reference cattle with subclinical Johne's disease (Table 3). The overall sensitivity (75%; Table 2) for subclinical disease was greater than those reported for the Allied ELISA, CSL ELISA, and complement fixation and agarose gel immunodiffusion tests (58.8, 43.4, 38.4, and 26.6%, respectively) (9, 39). The p35 recombinant protein was part of the crude lysate of the expressed product of the pMptb #40 recombinant clone, and it is possible that a serologic assay based on a purified p35 antigen might improve the ability to detect subclinical Johne's disease.

Recently, an a362 recombinant polypeptide of *M. paratuberculosis* (a purified portion of the 34K protein with B-cell epitopes which appear to be species specific) has been used in an ELISA for the diagnosis of Johne's disease. Although the reported sensitivity of the a362 ELISA was less than that of the p35 immunoblot assay (86%; Table 2) (9, 39), it is possible that combinations of purified p35 antigen, a362 peptide, and another potential specific antigen(s) or epitope(s) of *M. paratuberculosis* might provide improved serologic diagnosis. Such assays need to be investigated.

The 3.2-kb fragment of the pMptb #40 recombinant clone

hybridized only to DNAs from the *M. avium* complex, but not to DNAs from other mycobacterial and nonmycobacterial organisms. Although additional studies are needed, the data suggest that the 3.2-kb fragment has the potential of being used as a specific probe or used to design oligonucleotide primers for the development of an *M. avium*-specific PCR assay. The resurgence of *M. avium* complex disease in AIDS patients and the emergence of resistance of *M. avium* complex bacilli to multiple antimycobacterial drugs make the development of such an assay a useful tool for the specific and rapid differential detection of *M. avium* complex infections.

ACKNOWLEDGMENTS

We thank P. Brennan and Jill Clarridge for providing the bacterial strains. We are grateful to Diana Whipple, Mycobacteriosis Unit, National Animal Disease Center, Ames, Iowa; M. Collins, School of Veterinary Medicine, University of Wisconsin, Madison; Mitchell A. Yakrus, Mycobacteriology Laboratory Respiratory Disease Branch, Centers for Disease Control and Prevention; and Rod J. Chiodini, International Association of Paratuberculosis, Rehoboth, Mass., for providing serum samples used in this investigation.

This work was supported in part by the U.S. Department of Veterans Affairs and by the generous support of the Tom & Pat Powers Foundation.

REFERENCES

1. **Bech-Nielsen, S., J. B. Jorgensen, P. Ahrens, and N. C. Feld.** 1992. Diagnostic accuracy of a *Mycobacterium phlei*-absorbed serum enzyme-linked immunosorbent assay for diagnosis of bovine paratuberculosis in dairy cows. J. Clin. Microbiol. **30:**613–618.
2. **Braun, P. K., C. D. Buergelt, R. C. H. Littell, S. B. Linda, and J. R. Simpson.** 1990. Use of an enzyme-linked immunosorbent assay to estimate prevalence of paratuberculosis in cattle of Florida. J. Am. Vet. Med. Assoc. **196:**1251–1254.
3. **Buchanan, T. M., H. Nomaguchi, D. C. Anderson, R. A. Young, T. P. Gillis, W. J. Britton, J. Ivanyi, A. H. J. Kolk, O. Closs, B. R. Bloom, and V. Mehra.** 1987. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of *Mycobacterium leprae*. Infect. Immun. **55:**1000–1003.
4. **Chiodini, R. J., and J. M. Kreeger (ed.).** 1992. Proceedings of the Third International Colloquium on Paratuberculosis. International Association for Paratuberculosis Inc., Providence, R.I.
5. **Chiodini, R. J., and H. J. Van Kruiningen.** 1986. The prevalence of paratuberculosis in culled New England cattle. Cornell Vet. **76:**91–104.
6. **Chiodini, R. J., and W. C. Davis.** 1992. The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/elta T lymphocytes which prevent $CD4^+$ activity. Microb. Pathog. **13:**447–463.
7. **Chiodini, R. J., H. J. Van Kruiningen, and R. S. Merkal.** 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. **74:**218–262.
8. **Chiodini, R. J.** 1989. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. Clin. Microbiol. Rev. **2:**90–117.
9. **Cocito, C., P. Gilot, M. Coene, M. De Kesel, P. Poupart, and P. Vannuffel.** 1994. Paratuberculosis. Clin. Microbiol. Rev. **7:**328–345.
10. **Collins, M. T., N. Hoiby, J. B. Jorgensen, H. Berconier, R. S. Lambrecht, and E. Jorgensen.** 1991. Crossed immunoelectrophoretic analysis of *Mycobacterium paratuberculosis*. APMIS **99:**83–92.
11. **Douglas, M., J. Ford, J. Sanderson, S. Withey, M. Tizard, T. Doran, and J. Hermon-Taylor.** 1996. IS*900* PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cow's milk in England and Wales. Appl. Environ. Microbiol. **62:**3259–3264.
12. **El-Zaatari, F. A. K., L. Engstrand, D. C. Markesich, and D. Y. Graham.** 1992. Screening of a *Mycobacterium paratuberculosis* expression library with polyclonal antiserum and amplified DNA probes to identify species-specific immunodominant antigens, p. 254–268. *In* R. J. Chiodini and J. M. Kreeger (ed.), Proceedings of the Third International Colloquium on Paratuberculosis. International Association for Paratuberculosis, Inc., Providence, R.I.
13. **El-Zaatari, F. A. K., S. A. Naser, L. Engstrand, E. P. Burch, C. Y. Hachem, D. L. Wipple, and D. Y. Graham.** 1995. Nucleotide sequence analysis and seroreactivities of the 65K heat shock protein from *Mycobacterium paratuberculosis*. Clin. Diagn. Lab. Immunol. **2:**657–664.
14. **El-Zaatari, F. A. K., S. A. Naser, L. Engstrand, D. C. Markesich, D. C. Kalter, and D. Y. Graham.** 1996. Identification of *Mycobacterium avium* complex in sarcoidosis. J. Clin. Microbiol. **34:**2240–2245.
15. **El-Zaatari, F. A. K., S. A. Naser, L. Engstrand, C. Y. Hachem, and D. Y. Graham.** 1994. Identification and characterization of *Mycobacterium paratuberculosis* recombinant clones expressed in *E. coli*. Curr. Microbiol. **29:**177–184.
16. **Fifis, T., P. Plackett, L. A. Corner, and P. R. Wood.** 1989. Purification of a major *Mycobacterium bovis* antigen for diagnosis of bovine tuberculosis. Scand. J. Immunol. **29:**91–101.
17. **Harboe, M., R. N. Mshana, O. Closs, O. Closs, G. Kronvall, and N. H. Axelsen.** 1979. Cross-reactions between Mycobacteria. Scand. J. Immunol. **9:**115–124.
18. **Hermon-Taylor, J., M. Moss, M. Tizard, and J. Sanderson.** 1990. Molecular biology of Crohn's disease mycobacteria. Bailliere's Clin. Gastroentorol. **4:**23–42.
19. **Lisby, G., J. Andersen, K. Engbaek, and V. Binder.** 1994. *Mycobacterium paratuberculosis* in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction. Scand. J. Gastroenterol. **29:**923–929.
20. **McClure, H. M., R. J. Chiodini, D. C. Anderson, R. B. Swenson, W. R. Thayer, and J. A. Coutu.** 1987. *Mycobacterium paratuberculosis* (Johne's disease) in a colony of stump-tail macaques (*Macaca arctoides*). J. Infect. Dis. **155:**1011–1019.
21. **McKinzie, R. A., and W. H. Ward.** 1981. *Rhodococcus* (*Corynebacterium*) *equi*: a possible cause of reactions to the complement fixation for Johne's disease in cattle. Aust. Vet. J. **57:**200.
22. **Mehra, V., D. Sweetser, and R. A. Young.** 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA **83:**7013–7017.
23. **Moss, M., J. Sanderson, M. Tizard, J. Hermon-Taylor, F. A. K. El-Zaatari, D. Markesich, and D. Y. Graham.** 1992. PCR detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum* from long term cultures of Crohn's disease and control tissues. Gut **33:**1209–1213.
24. **Ridge, S. E.** 1993. Cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples by using elements of the Roche MB Check System. J. Clin. Microbiol. **31:**400–405.
25. **Roberts, G. D., E. W. Koneman, and Y. K. Kim.** 1991. Mycobacteria, p. 304–339. *In* A. Balows, W. J. Hausler, Jr., K. Herman, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **anderson, J. D., M. T. Moss, M. L. Tizard, and J. Hermon-Taylor.** 1992. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. Gut **33:**890–896.
28. **Sherman, D. M., J. M. Gay, D. S. Bouley, and G. H. Nelson.** 1990. Comparison of the complement fixation and agar gel immunodiffusion tests for the diagnosis of subclinical bovine paratuberculosis. Am. J. Vet. Res. **51:**461–465.
29. **Sockett, D. C., D. J. Carr, and M. T. Collins.** 1992. Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of *Mycobacterium paratuberculosis* infections in cattle. Can. J. Vet. Res. **56:**148–153.
30. **Sockett, D. C., T. A. Conrad, C. B. Thomas, and M. T. Collins.** 1992. Evaluation of four serological tests for bovine paratuberculosis. J. Clin. Microbiol. **30:**1134–1139.
31. **Sugden, E. A., B. W. Brooks, N. M. Young, D. C. Watson, K. H. Neilsen, A. H. Corner, C. Turcotte, A. Michaelides, and R. B. Stewart.** 1991. Chromatographic purification and characterization of antigens A and D from *Mycobacterium paratuberculosis* and their use in enzyme-linked immunosorbent assay for diagnosis of paratuberculosis in sheep. J. Clin. Microbiol. **29:**1659–1664.
32. **Thoen, C. O., and K. H. Baum.** 1988. Current knowledge on paratuberculosis. J. Am. Vet. Med. Assoc. **192:**1609–1611.
33. **Thoen, C. O., G. S. Colgrove, and L. D. Miller.** 1986. Johne's disease (paratuberculosis): a five- year experience in an infected herd, p. 483–486. *In* Proceedings of the 4th International Symposium on Veterinary Laboratory Diagnosis.
34. **Thole, J. R., H. G. Danwese, P. K. Das, D. G. Groothuis, L. M. Schouls, and J. D. A. Van Embden.** 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *E. coli*. Infect. Immun. **50:**800–806.
35. **Van Der Giessen, J. W. B., R. M. Haring, E. Vauclare, A. Eger, J. Haagsma, and B. A. M. Van Der Zeijst.** 1992. Evaluation of the abilities of three diagnostic tests based on the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in cattle: application in a control program. J. Clin. Microbiol. **30:**1216–1219.
36. **Vannuffel, P., P. Gilot, B. Lmibourg, B. Nearhuyzen, C. Dieterich, M. Coene, L. Machtelinckx, and C. Cocito.** 1994. Development of species-specific enzyme-linked immunosorbent assay for diagnosis of paratuberculosis. J. Clin. Microbiol. **32:**1211–1216.
37. **Wall, S., Z. M. Kunze, S. Saboor, I. Soufleri, P. Seechurn, R. Chiodini, and J. J. McFadden.** 1993. Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. J. Clin. Microbiol. **31:**1241–1245.
38. **Whipple, D. L., P. A. Kapke, and P. R. Andesen.** 1992. Comparison of a commercial DNA probe test and three cultivation procedures for the detection of *Mycobacterium paratuberculosis* in bovine feces. J. Vet. Diagn. Invest. **4:**23–27.
39. **Young, R. A., B. R. Bloom, C. M. Crosskimsky, J. Ivanyi, D. Thomas, and R. W. Davis.** 1985. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA **82:**2583–2587.