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Detection of Cilia-Associated Respiratory Bacillus by PCR

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The cilia-associated respiratory (CAR) bacillus is an unclassified, gram-negative, motile bacterium that has been implicated as an etiologic agent of respiratory disease in laboratory rodents. In the present study, approximately 1,200 bases of the 16S rRNA gene from three CAR bacillus isolates were sequenced. CAR bacillus-specific primers were designed on the basis of the 16S rRNA gene sequence and used in a PCR assay. The PCR assay detected as little as 500 fg of purified CAR bacillus DNA. The expected 267-bp DNA fragment was amplified from respiratory tissue of frozen, formalin-fixed, and paraffin-embedded samples from experimentally and naturally infected rats and mice. In contrast, no product was amplified from respiratory tissues of sham-infected experimental animals or animals that were serologically or histopathologically negative for the CAR bacillus. Our findings indicate that this PCR assay is a rapid, specific, and sensitive detection method for the diagnosis of CAR bacillus infection in rats and mice.

The cilia-associated respiratory (CAR) bacillus is an unclassified, gram-negative, motile, non-spore-forming bacterium initially recognized in 1980 in lungs of rats with pneumonia (19). Since that time, CAR bacillus infections of laboratory rats and mice have been correlated with increased morbidity and mortality (3, 6, 8, 11). Clinical signs may include weight loss, a rough hair coat, and wheezing, but often CAR bacillus infections are asymptomatic (4, 17, 18). The significance of subclinical infection with the CAR bacillus has yet to be determined; however, the close association of the organism with the ciliated respiratory epithelium raises concerns about its potential effects on the ciliary functions of infected animals (12). Identification of subclinically infected laboratory animals is critical, since the use of infected animals may result in altered or invalid research findings, particularly in studies that examine respiratory function or response.

Several testing methods are used to diagnose CAR bacillus infections, including histology, serology, and bacteriology. Diagnosis of CAR bacillus infection is routinely accomplished by histologic examination of respiratory tissues to detect the bacteria between cilia of the respiratory epithelium. However, the bacteria are difficult to distinguish from cilia when stained with conventional stains, such as hematoxylin and eosin. Special stains, such as Warthin-Starry and Steiner silver stains, are required to stain the bacillus differentially (4; unpublished results). Alternatively, enzyme-linked immunosorbent assays (ELISAs) have been developed to diagnose infected animals by detecting the presence of serum antibody to the CAR bacillus (4, 9). Unfortunately, cross-reactivity with antibodies to other bacterial species limits the specificity of currently used ELISAs. In vitro culture of the CAR bacillus is the definitive method for identification of infected animals. This technique, however, is expensive and labor-intensive because this fastidious bacterium must initially be propagated in either embryonated eggs or mammalian cell cultures (4, 7). Diagnosis via culture is slow, often requiring 10 to 14 days before CAR bacillus growth can be detected. In addition, contamination of cultures with commensal bacteria is common, further hindering diagnosis. Given the limitations of the available diagnostic methods, a rapid, direct method for detection of CAR bacillus infections in laboratory animals is needed.

In this study, gene amplification was investigated as a method for diagnosis of CAR bacillus infection in laboratory rats and mice. The 16S rRNA gene from three CAR bacillus isolates obtained from infected rats was sequenced, and a consensus sequence was determined. Primer sequences were selected within the 16S rRNA gene which were unique to the CAR bacillus but conserved among the three isolates sequenced. Primers were synthesized and a DNA amplification procedure was developed to detect the CAR bacillus in frozen, formalin-fixed, and paraffin-embedded tissues from experimentally and naturally infected laboratory animals. The results obtained indicate that this PCR assay is a rapid, specific, and sensitive method for identification of CAR bacillus-infected rodents.

MATERIALS AND METHODS

Bacterial isolates and cultivation. Three CAR bacillus isolates from naturally infected rats were obtained from geographically distinct sources and designated R1, R2, and R3. Isolates were collected by tracheal lavage of infected rats anesthetized with an intramuscularly administered dose of xylazine (10 mg/kg) mixed with ketamine hydrochloride (50 mg/kg). The trachea was aseptically exposed and lavaged with 0.4 ml of sterile phosphate-buffered saline (PBS), pH 7.2. The tracheal aspirate was used to inoculate subconfluent mouse 3T3 fibroblasts grown in Dulbecco modified Eagle medium (Hazleton, Lenexa, Kans.) supplemented with 10% fetal bovine serum (Hazleton) and 2% L-glutamine (Sigma, St. Louis, Mo.). After incubation at 37°C for 48 h, mouse 3T3 fibroblast monolayers were scraped to detach the majority of mammalian cells and the medium was removed. Fresh medium was added, and incubation was continued at 37°C until CAR bacillus concentrations reached approximately 10^5 to 10^6 bacteria per ml, as determined by quantitation of bacteria with a hemacytometer viewed by phase-contrast microscopy. To harvest the CAR bacillus, the flasks were scraped and the medium was centrifuged at 1,000 × *g* for 20 min at 4°C to remove the remaining 3T3 fibroblasts. The supernatant fraction was cen-

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TABLE 1. Oligodeoxyribonucleotide primers used to amplify the 16S rRNA gene sequence of the CAR bacillus

Primer	Sequence (5′ to 3′)	Position[a]
Broad-range prokaryotic		
g2	GCTTAACACATGCAAGTCGAA	46–68
cg4	GGTGGACTACCAGGTATCTAATCC	810–785
p93E	CCGCACAAGCGGTGGAGCA	930–950
13B	AGGCCCGGGAACCTATTCAC	1390–1371
CAR bacillus		
gap403	GGTGTAGGGGTAAAATCCGTAG	403–424
RF141	GGGGGAAAGAAACCTAGTTTT	141–161
RR408	ACACCCTTAGAAAAGGGGATT	407–387

[a] The position within the *E. coli* 16S rRNA gene sequence that corresponds to the 5′ and 3′ ends of each primer is shown. Approximate positions are given for CAR bacillus-specific primers.

trifuged at 10,000 × *g* for 30 min at 4°C to pellet the bacteria. The pellet was washed three times with PBS, pelleted by centrifugation, and stored at −20°C until used.

Isolation of bacterial DNA. DNA was extracted and purified from CAR bacillus pellets by using a modification of the technique described by Marmur and Doty (10). Briefly, bacteria were lysed by enzymatic digestion with lysozyme (10 mg/ml) on ice for 45 min. The protein fraction was solubilized with proteinase K (1 mg/ml) and sodium dodecyl sulfate (0.5%) overnight at 37°C. Nucleic acids were extracted with phenol-chloroform and precipitated with ethanol. DNA preparations were resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 8.0), treated with RNase (200 μg/ml) overnight at 4°C, extracted with chloroform, and reprecipitated with ethanol. The resulting chromosomal DNA preparations were dissolved in TE and stored at −20°C until used.

In addition, DNAs were isolated as described above from the following organisms: *Klebsiella pneumoniae*, *Escherichia coli*, *Pasteurella multocida*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pasteurella pneumotropica*, and *Mycoplasma pulmonis*. Purified DNAs from these respiratory bacteria were used to test the specificity of the CAR bacillus PCR assay.

Oligonucleotide primers. Oligonucleotide primers (Table 1) were synthesized at the DNA Core Facility, University of Missouri, Columbia. The sequences of broad-range prokaryotic primers (g2, cg4, p13B, and p93E) were adapted from previous studies (2, 16). CAR bacillus-specific primers gap403, RF141, and RR408 were designed on the basis of the 16S rRNA gene sequence determined in this study.

PCR amplification. Reactions were performed in a 100-μl volume. Each reaction mixture contained various amounts of template DNA, each oligodeoxyribonucleotide primer at 1 μM, 67 mM Tris (pH 8.0), 16.6 mM ammonium sulfate, 5 mM magnesium acetate, each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) at 1.25 mM, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Reactions were performed in an automated, programmable Coy Thermocycler (Coy Corporation, Green Lake, Mich.). PCR cycles consisted of 1 min of denaturation at 94°C, 1 min of annealing, and 2 min of extension at 72°C for 35 cycles. The annealing temperature for primers g2 and cg4 was 50°C; the annealing temperature for all other primers was 55°C. PCR products (10 μl) were electrophoretically separated in a 3% NuSieve agarose gel (FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and visualized by UV light. DNA markers of known sizes were run on each gel to facilitate determination of the sizes of the reaction products.

To test the sensitivity of the PCR assay, 10-fold serial dilutions of purified CAR bacillus DNA, ranging from 50 ng to 50 fg, were added to standard PCRs containing 5 μg of DNA isolated from respiratory tissue of uninfected animals. Amplification reactions were cycled. Products were subjected to electrophoresis and visualized as described above.

DNA sequencing and analysis. Amplified products were purified on 3.5% polyacrylamide gels, and the sequences were determined by the *Taq* dideoxy-chain termination method with a commercially available kit (*Taq* Dye Deoxy Terminatory Cycle Sequencing Kit; Applied BioSystems, Inc., Foster City, Calif.). Sequence data were analyzed with the EuGene software package (Baylor College of Medicine, Houston, Tex.).

Determination of G+C content. To complement the 16S rRNA sequence analysis, the G+C contents (in moles percent) of the purified chromosomal DNAs of all three rat origin CAR bacillus isolates were determined. Determinations were performed by C. W. Gehrke and K. C. Kuo (Station Chemical Laboratories, University of Missouri, Columbia) with high-performance liquid chromatography methods (5).

Animal infections. Ten adult outbred albino rats from an in-house colony known to be endemically infected with the CAR bacillus and 12 adult Sprague-Dawley rats from a CAR bacillus-negative colony (Harlan Sprague Dawley, Indianapolis, Ind.) were obtained. The infection status of the colonies had previously been documented by histologic and serologic evaluations. Rats to be tested by PCR were bled for serologic evaluation of CAR bacillus infection and necropsied. Portions of the respiratory tissues were frozen at −80°C for DNA isolation and PCR amplification. To assess the most appropriate tissue for PCR analysis, samples of different respiratory tissues (nasoturbinates, tracheas, and lung lobes) were also frozen separately for subsequent PCR assay. Sera were tested by ELISA for CAR bacillus antibody as described above.

Twelve 2-week-old BALB/c mice were obtained from a colony proven to be histologically and serologically negative for the CAR bacillus (National Cancer Institute Frederick Cancer Research Developmental Center, Frederick, Md.). Six pups were inoculated intranasally with approximately 10^5 CAR bacilli (isolate R3) suspended in 50 μl of PBS. The remaining six pups were inoculated with 50 μl of PBS and served as controls. At 8 weeks postinoculation, the animals were euthanized, bled for serologic evaluations, and necropsied. Transtracheal lavage fluids were collected and used to inoculate mammalian cell cultures for isolation of CAR bacilli. Tracheas and lungs were removed, and portions of each were frozen at −80°C until used. The remaining tissues were preserved in 10% neutral buffered formalin for 48 h. Formalin-fixed tissues were paraffin embedded and sectioned for silver staining and PCR amplification. Sera were subjected to ELISA to detect CAR bacillus antibody at the Research Animal Diagnostic and Investigative Laboratory, Columbia, Mo.

Isolation of DNA from tissue samples. Paraffin-embedded respiratory tissues from 17 naturally infected rats, three infected mice, 10 uninfected rats, and three uninfected control mice were obtained from the archives at the Research Animal Diagnostic and Investigative Laboratory. Archival tissue samples had been stored for 1 to 6 years as paraffin-embedded blocks. CAR bacillus infection status had previously been proven by histologic and serologic evaluations. Tissues most often consisted of nasoturbinates, tracheas, and/or left lung lobes.

Frozen tissues were cut into 2- to 3-mm cubes, minced, resuspended in 1 ml of TE, boiled for 10 min, and allowed to cool to room temperature prior to amplification by PCR. Paraffin-embedded tissues were processed as described by

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GCGCCGTGCA GTGTAGCATT CCACTGGCGG CGACCGGCAA ACGGGTGCGT AACGCGTACA
CAACTTGCCT TTGAGTGGAG CATAACACCG AGAAATTGGT GCTAATTCTC CATAGTATTG
CTGAAGGGAG TCCTTTGGTA ATTAAAGCTC CGGCGCTCGA AGATGGGTGT GCGTCCGATT
AGATAGTCGG TGAGGTAACG GCTCACCGAG TCTACGATCG GTAACTGGTG TGAGAGCACG
ACCAGTCACA CGGACACTGA GACACGGGTC CGACTTCTAC GGGAGGCAGC AGTGAGGAAT
ATTGGTCAAT GGGCGCAAGC CTGAACCAGC CATGCCGTGT GAAGGATGAA GACTCTCAGA
GTCGTAAACT TCTTTTATTT GGAATGAAAT CCCCTTTTCT AAGGGTGTTG ACAGTACCAT
TTGAATAAGC ACCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG GTGCGAGCGT
TATCCGGATT TATTGGGTTT AAAGGGTGTG CAGGCGGGTA GGTAAGTCGA TTGTGAAATC
CCCGAGCTTA ACTCGAGAAT TGCATTCGAT ACTGCCTATC TTGAATACTG CGGAGGTGAG
TAGAATATGT CATGTAGCGG TGAAATGCTC AGATATGACA TAGAATTCCG ATTGCGAAGG
CAACCTACTG GAACATTACT GACGCTCATG TGCGAAAGCG TGGGGAGCAA ACAGGATTAG
ATACCCTGGT AGTCCACGCC CTAAACGATG AATGCTAGTT GTTGGGGTGC TTGTCACTCC
AGTGATGCAG CTAACGCATT AAGCATTCCG CCTGGGGAGT ACGATCGCAA GATTAAAACT
CAAAGGAATA GACGGGGACC CGCACAAGCG GTGGAGCATC TGGTTTCATT CGATGATACG
CGAGGAACCT TACCTAGGCT AGAATGTCGT TGGACATTCC CTGAATGGGG GTCTTGTAGC
AATACACTGA CTGGAAGGTA GTGCATGGAT GTCGTCAGCT CGTTCCGTTA GGTGTAGGTT
TAAGTCCCGC ACCGAGCGCA ACCCCCCTTC TTTGTTGGCA GCTAGTAAAG TCGGGAACTC
TGACAAAACT GACGTCCCAC GGTGTGAGGA GGGAGGGGAT GATGTCAAGA CATCATCGTC
TTCATGCCTA GGGGTACACA AGANTTACAA TAGGTGCTAC AGCAGGTCGC GACATAGCAA
TATGGAGCTA ANCTCCAAAA GCACTCTCAG TTCGGCTTGT AGG
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FIG. 1. Consensus sequence generated from the positive strand of the 16S rRNA gene of three rat origin CAR bacillus isolates.

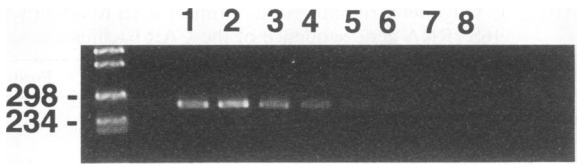

FIG. 2. PCR amplification products from various amounts of template DNA. This ethidium bromide-stained gel shows PCR products resulting from amplification of serial dilutions of CAR bacillus DNA in the presence of respiratory DNA from uninfected rats. Lanes: 1, no-template control; 2, 50 ng of template DNA; 3, 5 ng of template DNA; 4, 500 pg of template DNA; 5, 50 pg of template DNA; 6, 5 pg of template DNA; 7, 500 fg of template DNA; 8, 50 fg of template DNA. Migration of molecular size markers is shown in the leftmost lane. Sizes are in base pairs.

Relman et al. (15). Ten 5-μm sections from paraffin-embedded blocks of tissue were deparaffinized in two washes of xylene and washed twice with absolute ethanol. Residual ethanol was removed by vacuum desiccation. One hundred microliters of digestion buffer (50 mM Tris [pH 8.5], 1 mM EDTA [pH 8.0], 0.5% Tween 20, 200 μg of proteinase K per ml) was added to each tube, and mixtures were heated at 55°C. After 3 h, the proteinase K was inactivated by incubation at 95°C for 10 min. A 5-μl aliquot of each DNA preparation was subjected to PCR amplification as described above.

Nucleotide sequence accession number. The sequence reported here has been assigned GenBank accession number U09452.

RESULTS

Sequence of the 16S rRNA gene. Regions of the 16S rRNA gene from three CAR bacillus isolates were amplified with broad-range prokaryotic primers (Table 1), and the nucleotide sequences were determined. On the basis of these data, an additional oligonucleotide primer, gap403, was synthesized to construct overlapping fragments of the 16S rRNA gene and the resulting amplified fragment was also sequenced. By this means, a consensus sequence of 1,243 contiguous base pairs of the 16S rRNA gene was determined for three rat origin CAR bacillus isolates (Fig. 1). The sequence homology among the three isolates was 99.4%.

Phylogeny of the CAR bacillus. Analysis of the 1,243-bp 16S rDNA consensus sequence from rat origin CAR bacillus isolates suggested that the bacteria may belong to a subdivision of gram-negative gliding bacteria. Comparison of the sequence in the GenBank database indicated a distant relationship to *Flavobacterium ferruginum* (77% similarity), *Microscilla furvescens* (73%), *Flexibacter polymorphus* (73%), and *Cytophaga fermentans* (72.9%). The mean G+C content determined for the three CAR bacillus isolates was 38.17 ± 3.08 mol%.

Evaluation of PCR primers specific for the CAR bacillus. Sequence analysis was used to design two CAR bacillus-specific primers, RF141 and RR408, that corresponded to regions of the 16S rRNA sequence unique to the CAR bacillus but conserved among the three isolates sequenced (Table 1). Amplification of DNAs from all three in vitro-grown rat origin CAR bacillus isolates with CAR bacillus-specific primers yielded the expected 267-bp product. The specificity of the CAR bacillus-specific primers was tested in PCRs with DNAs from seven other respiratory bacteria found in laboratory animals, including *K. pneumoniae*, *E. coli*, *P. multocida*, *P. mirabilis*, *P. aeruginosa*, *P. pneumotropica*, and *M. pulmonis*. The expected 267-bp product was not generated when DNAs from these organisms were used in PCR assays with CAR bacillus-specific primers (data not shown).

To assess the sensitivity of the assay, serial 10-fold dilutions of CAR bacillus DNA were used in PCRs containing DNA isolated from respiratory tissues of uninfected animals. An amplified product of the expected size was detected in ethidium bromide-stained gels when as little as 500 fg of CAR bacillus DNA was used as the template in amplification reactions (Fig. 2).

PCR of DNA from tissue samples. To determine the most appropriate tissues for detection of CAR bacillus infection in laboratory rodents, DNA was isolated from various portions of frozen respiratory tissues and subjected to PCR with CAR bacillus-specific primers. The CAR bacillus product was consistently amplified from trachea and lung lobe tissues but not from nasoturbinate tissues (Fig. 3).

To determine the utility of this assay for the diagnosis of CAR bacillus infections in rodents, DNA amplification with CAR bacillus-specific primers was performed on frozen respiratory tissue samples obtained from naturally and experimentally infected animals. The infection status of all animals was confirmed histopathologically by evaluation of respiratory tissue for the presence of cilia-associated bacteria and/or serologically by the presence of serum antibody to the bacillus. An amplified product of the expected size was detected in 10 of 10 infected rats and six of six infected mice. In contrast, no PCR products were detected in any of the 12 uninfected rats or the six uninfected mice.

The feasibility of using the PCR assay to amplify DNA from formalin-fixed and formalin-fixed, paraffin-embedded tissues

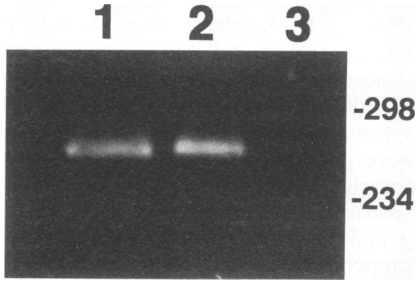

FIG. 3. PCR amplification products from various tissues. This ethidium bromide-stained gel shows PCR products resulting from amplification of DNA from various infected respiratory tissues with CAR bacillus-specific primers. Lanes: 1, trachea; 2, lung; 3, nasoturbinate. Migration of molecular size markers is shown on the right. Sizes are in base pairs.

TABLE 2. Detection of CAR bacillus by amplification of frozen, formalin-fixed, and paraffin-embedded tissues from infected rodents

Animal group	No. of positive samples/total no. tested		
	Frozen	Formalin fixed	Paraffin embedded
Infected			
Experimental infections	6/6	6/6	6/6
Natural infections	10/10	10/10	10/10
Archival	ND[a]	ND	20/20
Uninfected			
Experimental infections	0/6	0/6	0/6
Natural infections	0/12	0/12	0/12
Archival	ND	ND	0/13

[a] ND, not done.

was assessed by using appropriately processed tissue samples from infected animals and uninfected control animals. Results were compared with PCR results obtained with identical samples that were frozen and PCR amplified (Table 2). Representative results are shown in Fig. 4 along with amplification results obtained with analogous frozen tissue samples. The expected 267-bp PCR product was amplified in both formalin-fixed and paraffin-embedded tissue samples from rats and mice with documented histological and serological evidence of CAR bacillus infection. In contrast, evaluation of PCR results from formalin-fixed and paraffin-embedded respiratory tissues of rodents with no histologic or serologic evidence of CAR bacillus infection revealed no detectable amplification products. Paraffin-embedded archival tissue samples which had been stored for 1 to 6 years were also evaluated by this PCR assay (Table 2). Amplification results showed an absolute correlation with the infection status previously documented by histopathologic evaluation. Of the 20 archival samples from infected animals, 3 were obtained from naturally infected mice. DNA amplification of mouse origin CAR bacillus sequences by primers designed on the basis of rat origin CAR bacillus sequences demonstrated the potential of this PCR assay for use in the diagnosis of CAR bacillus infections in mice.

DISCUSSION

In this study, the suitability of DNA amplification for detection of CAR bacillus infections in infected rats and mice was examined. Oligonucleotide primers specific for the CAR bacillus were synthesized on the basis of nucleotide sequence

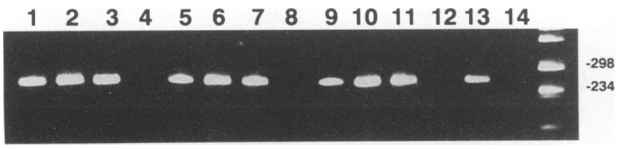

FIG. 4. Amplification of CAR bacillus sequences from DNA preparations of frozen, formalin-fixed, and paraffin-embedded tissues obtained from naturally infected rats and mice. Lanes: 1 to 3, frozen tissues from infected rats; 4, frozen tissue from an uninfected rat; 5 to 7, formalin-fixed tissues from infected rats; 8, formalin-fixed tissue from an uninfected rat; 9 to 11, paraffin-embedded tissues from infected rats; 12, paraffin-embedded tissue from an uninfected rat; 13, paraffin-embedded tissue from an infected mouse; 14, paraffin-embedded tissue from an uninfected mouse. Migration of molecular size markers is shown in the rightmost lane. Sizes are in base pairs.

analysis of the 16S rRNA gene from three CAR bacillus isolates obtained from naturally infected rats. PCRs were tested by using these primers and template DNA obtained from respiratory tissues of animals whose CAR bacillus infection status was known. The expected-size product was amplified from tissues of infected animals but not from samples from uninfected animals. Successful amplification of frozen, formalin-fixed, and paraffin-embedded tissues indicates that tissues processed by any of these methods can be used to detect CAR bacillus infections in rodents. However, it is important to note that extended formalin fixation times can be detrimental to the efficiency of PCR amplification (1).

Examination of various respiratory tissues in PCR assays demonstrated that CAR bacillus sequences were amplified from trachea and lung lobe tissues but not from nasoturbinate tissue. The failure to obtain an amplified product from nasoturbinate samples was disappointing. Since the nasoturbinates are the first sites of CAR bacillus colonization (4, 12), this would have been an ideal tissue for early detection of subclinical infections. The failure of PCR to detect the CAR bacillus cannot be explained by limited numbers of organisms, since histologic examination of nasoturbinates from infected rodents revealed large numbers of the bacteria. However, it is possible that DNA preparations from nasoturbinates may have a high degree of protein contamination and/or contain inhibitors that interfere with the PCR (1). Additional modification of the sample preparation protocol may be needed to enhance DNA amplification of nasoturbinate samples.

The specificity of the PCR assay was demonstrated as tissues tested from infected rodents were positive by amplification, whereas analogous tissues from noninfected rats and mice were negative. The specificity of PCR was also shown by its failure to amplify the target sequence in reactions with DNAs from other representative bacterial species, indicating that the primer sequences used represent unique sequences in the rat origin CAR bacillus. Little is known about CAR bacillus isolates from mice; i.e., a CAR bacillus isolate has not been cultured from infected mice, and sequence information is not available. Thus, mouse and rat origin isolates have not been biochemically or genetically compared. Successful amplification of CAR bacillus sequences in mouse tissues by using primers based on the sequences obtained from rats suggests that the mouse origin isolates used in this study are closely related to rat origin isolates in that at least a portion of the 16S rRNA gene is conserved.

With the PCR assay, we were able to detect a DNA product when as little as 50 fg of purified CAR bacillus DNA was amplified. Although our data showed no qualitative differences in amplification of frozen, formalin-fixed, or formalin-fixed, paraffin-embedded tissue samples, the sensitivity of the PCR assay may be less in formalin-treated samples because of cross-linking caused by formalin.

The sequence of the 16S rRNA gene has been used to study phylogenetic relationships. Similarity among the 16S rRNA sequence data indicates that the rat origin CAR bacillus isolates are distantly related to representatives of the genus *Flavobacterium* and the order *Cytophagales*, which confirms recent taxonomic studies by Schoeb and coworkers (16). Phylogenetic analyses of gliding bacteria indicate that bacteria within this group are heterogenous and probably represent many different genera (13, 14). Additional information, including results of DNA-DNA hybridization studies, is needed to place the CAR bacillus within a genus and species.

In summary, specific primers for the rat origin CAR bacillus isolate amplified a unique 267-base sequence within the 16S rRNA gene in infected tissues but not in uninfected tissues.

The expected fragment was successfully amplified in frozen, formalin-fixed, and paraffin-embedded tissues from experimentally and naturally infected mice and rats. Results of this study indicate that this PCR assay may be useful as a diagnostic tool for rapid detection of CAR bacillus infection in rats and mice.

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