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Detection of *Mycoplasma pulmonis* in Cilia-Associated Respiratory Bacillus Isolates and in Respiratory Tracts of Rats by Nested PCR

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To improve the detection of *Mycoplasma pulmonis* contamination of isolates of cilia-associated respiratory (CAR) bacillus, we developed a nested PCR method using primers for 16S rRNA gene sequences. Of 140 samples of 16 different CAR bacillus isolates, 73 (52%) were inhibitory in the first PCR, as indicated by the absence of amplicons of the internal control, but only 11 of 140 (7.9%) were inhibitory in the second PCR. Of 27 samples known to contain *M. pulmonis*, only 12 (44%) were positive in the first PCR, but 25 of 27 (93%) were positive in the second PCR. Nested PCR also detected *M. pulmonis* in 21 of 61 (34%) CAR bacillus samples from which *M. pulmonis* could not be cultured and identified 2 additional *M. pulmonis*-contaminated CAR bacillus isolates. Of 359 respiratory and reproductive tract lavage samples from rats and mice, 35 (9.8%) were inhibitory in the first PCR, but only 15 (4.2%) were inhibitory in the second PCR. Of 72 lavage specimens from rats inoculated with an avirulent, poorly infective *M. pulmonis* strain, 14 (19%) were positive by nested PCR, but only 2 of 72 (2.8%) were positive by culture. Nested PCR also detected *M. pulmonis* in 14 of 20 (70%) paraffin sections of lung and trachea from rats and mice inoculated with CAR bacillus isolates known to contain *M. pulmonis*, whereas single PCR gave no positive results. We conclude that nested PCR is superior to single PCR or culture for detecting *M. pulmonis*, and that *M. pulmonis* is present in all but four CAR bacillus isolates in our collection that were from naturally infected rats; the four isolates that were exceptions were obtained from rats from a single colony.

Cilia-associated respiratory (CAR) bacillus of rats is a gram-negative, gliding, and flexing filamentous rod that parasitizes the respiratory tract (17). It is unclassified, but it is known to be related to flavobacteria (3, 16). It causes respiratory disease closely similar to that caused by *Mycoplasma pulmonis* (14, 17) and is a potential complication of research in which rats are used, although little is known concerning the pathogenesis, significance, or prevalence of CAR bacillus infection. CAR bacillus and *M. pulmonis* infections are commonly concurrent in animals with naturally occurring disease; consequently, many CAR bacillus isolates from naturally infected rats are contaminated with *M. pulmonis* (17). It is therefore crucial that CAR bacillus isolates to be used for experimental infections be rigorously determined to be free of *M. pulmonis*, but such contamination can be difficult to detect (16). It is also necessary that rats experimentally infected with CAR bacillus be demonstrably free of *M. pulmonis* before and after inoculation. Although *M. pulmonis* infection usually can be detected by culture and enzyme-linked immunosorbent assay, the organism can be difficult to isolate from animals with mild or subclinical infections, and rats with such infections may not develop diagnostic concentrations of serum antibody until they are several months old (2). A further complication is that infections with other rodent mycoplasmas, such as *Mycoplasma arthritidis* and *Mycoplasma collis*, can evoke cross-reacting antibodies and false-positive results in enzyme-linked immunosorbent assays for *M. pulmonis* antibodies (2, 4, 5).

Detection of *M. pulmonis* by PCR offers the potential to circumvent these difficulties, and several PCR methods have been described (7, 9, 10, 19). However, the efficiency of PCR can be reduced with some types of samples and sample preparation methods, often due to various impurities that may not be removed by routine extraction methods (13). In some cases, inhibition of amplification can occur in one-third or more of the reactions (18). We found that samples from CAR bacillus cultures frequently were inhibitory to amplification of *M. pulmonis* target sequences, such that even samples from which *M. pulmonis* had been cultured gave negative results by single PCR (16). A variety of PCR modifications, product detection methods such as blotting and use of radiolabeled probes, and sample preparation methods such as use of glass powder or spin columns can improve PCR results, although none is successful in all situations. Nested PCR is one technique that can provide improved results with inhibitory samples. Unless a sample is so inhibitory as to prevent amplification completely, the first PCR provides at least some increase in the amount of template available for the second PCR. In addition, amplification efficiency can be improved as a result of the reduction in the concentration of inhibitors due to dilution of the sample during preparation of the second reaction mixture. We found that a nested PCR method with primers for 16S rRNA gene sequences worked well for the detection of *M. pulmonis*. Each PCR incorporated an internal control, and dot blotting and probing with an oligonucleotide probe for a sequence unique to *M. pulmonis* were used as final criteria of specificity. We found that *M. pulmonis* was present in most CAR bacillus

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isolates, including two isolates, one from a rat and one from a rabbit, from which *M. pulmonis* could not be cultured. *M. pulmonis* also was detected in nasal lavage specimens from rats inoculated with an avirulent, poorly infective strain and in formalin-fixed, paraffin-embedded sections of tissues from rats inoculated with CAR bacillus isolates known or suspected to be contaminated with *M. pulmonis.*

MATERIALS AND METHODS

CAR bacilli. CAR bacillus strains were designated according to their sources (16). CAR bacillus NIH, the reference strain, and CAR bacillus StL were derived from the original isolate obtained in embryonated eggs by Ganaway et al. (6). CAR bacilli isolated in our laboratory were designated according to their accession numbers. Strains X1247C, X1331B, X1428D, X2450D, X2450E, X2450F, and X2450G were isolated from naturally infected rats, and strain X2006C was cultured from a naturally infected rabbit. Strains X1403B, X1404B, X1328E, X1328F, and X1328I were isolated from rats or mice inoculated with CAR bacillus NIH. CAR bacillus X1428D/AS was derived from CAR bacillus X1428D by treatment with hyperimmune rabbit serum against *M. pulmonis* (14). CAR bacilli NIH and StL were grown on BALB 3T3 cells when they were originally received and were subsequently grown as described previously (16) in Dulbecco's minimal essential medium (DMEM; Sigma Chemical Co., St. Louis, Mo.) with 10% fetal calf serum (HyClone Laboratories, Logan, Utah) or DMEM and Coon's modified Ham's F12 medium (1:1; DMEM-F12 medium; Sigma) with 10% fetal calf serum. Other strains were originally isolated in DMEM or DMEM-F12 and were subsequently grown in DMEM-F12.

Samples and sample preparation. CAR bacillus samples included culture medium from CAR bacillus cultures, freshly grown CAR bacilli alone, or both. Samples were centrifuged at 14,000 × *g* for 15 min at 4°C, and the resulting pellets were prepared either by chloroform-phenol extraction or by treatment with lysis buffer containing 1% Tween 20, 1% Triton X-100, and 0.5 mg of proteinase K per ml and incubation at 60°C for 1 h, followed by incubation at 95°C for 10 min to inactivate the proteinase K. The volume of lysis buffer ranged from 50 μl for minimal pellets to 200 μl for substantial (approximately 25- to 50-μl) pellets of CAR bacilli. Lavage samples were obtained from the respiratory tracts of rats and mice with natural or experimental CAR bacillus infection by flushing the nasal passages or trachea, bronchi, and lungs with nonenzymatic cell dissociation buffer (Sigma). One milliliter of buffer was used for nasal lavages of both rats and mice; lungs of mice were lavaged with up to 3 ml of buffer and those of rats were lavaged with up to 15 ml. Samples of liquid mycoplasma medium were from cultures of CAR bacillus NIH or from respiratory tract cultures from rats or mice that had been inoculated with CAR bacillus NIH up to 12 weeks previously; weak color changes occurred in these samples, but no organisms could be subcultured on solid mycoplasma media. Cultural procedures and the formulation of SP-4 and A media were as described previously (16). Lavage and mycoplasma culture samples were prepared by centrifugation and treatment with lysis buffer as described above. Sections of trachea or lung, or both, were from rats or mice that had been inoculated with CAR bacilli up to 12 weeks previously and were prepared by fixation in 10% neutral buffered formalin for 2 to 7 days followed by routine embedding in paraffin and sectioning. Sections were processed as described previously (20). Two sections of each block were deparaffinized with xylene, rehydrated, and incubated overnight at 37°C in digestion buffer (pH 8.0) containing 50 mM Tris, 1 mM EDTA, 0.5% Tween 20, and 200 μg of proteinase K per ml. After digestion, the samples were heated to 95°C for 10 min and centrifuged briefly to sediment undigested tissue fragments.

PCR. Primers for sites in the first three variable regions of the 16S rRNA gene of *M. pulmonis* (GenBank accession no. M23941) were designed with MacVector software (International Biotechnologies, Inc., New Haven, Conn.). The external primers Mp.F3 and Mp.R1 produce a 402-bp amplicon and have the respective sequences 5′-TGCTT AGCGG CAAAT GGGTG-3′ (nucleotide positions 88 to 107 according to *Escherichia coli* numbering) and 5′-GGTAC CGTCA TACTT AGGG-3′ (nucleotide positions 471 and 489). The internal primers Mp.F1 and Mp.R2 produce a 283-bp product and have the respective sequences 5′-CTGTT GCTAA TACCG GATAT GT-3′ (positions 156 to 177) and 5′-ACAGC AGTTT ACAAT CCGAA GACC-3′ (positions 416 to 439). Oligonucleotides were synthesized by the DNA Synthesis Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research. Each PCR was optimized for Mg^{2+} concentration, annealing temperature, and primer concentration with internal controls included in the reaction mixture (13). For both first and second PCRs, the concentrations in a 50-μl reaction mixture were as follows: primers, 300 nM; deoxynucleoside triphosphates, 200 μM; *Taq* polymerase, 2.5 U/100 μl; and Mg^{2+}, 2.0 mM. Sample volumes of 1 to 3 μl were used in the first PCR; smaller sample volumes were used with samples derived from pelleted CAR bacilli, inasmuch as these contained large amounts of background DNA. The temperature cycle for the first PCR was denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, for 40 cycles. The temperature cycle for the second PCR was the same, except that the annealing temperature was 60°C. Reaction mixtures for the second PCR were prepared with reaction mixtures from the first PCR at 1/10 of the total volume (5 μl in a 50-μl reaction mixture). The products were visualized by gel electrophoresis of 25-μl samples of the reaction mixtures in 2% NuSieve and ethidium bromide staining. Strict measures to control contamination were used, including advance preparation of single-use aliquots of reaction mixtures and other reagents, exclusive use of aerosol-resistant pipet tips, and use of UV-equipped class II laminar flow hoods for all procedures. A negative control was included with each set of reactions. Internal controls for each PCR were constructed with Mimic kits (Clontech, Palo Alto, Calif.) so as to have *M. pulmonis* primer sequences flanking a 556-bp segment of unrelated DNA, resulting in mimics of 595 and 602 bp for the first and second PCRs, respectively. Each reaction mixture included 100 aM of the respective mimic. Thus, a reaction giving bands at the expected 600-bp position was interpreted as not significantly inhibited and therefore giving a valid negative result, within the limits of sensitivity, if no *M. pulmonis* product was evident. If no product was evident either for the internal control band or for target *M. pulmonis* sequences, the reaction was interpreted as inhibited. Samples giving bright bands for the *M. pulmonis* target sequence but no internal control band were interpreted to contain enough *M. pulmonis* target that the internal control was not amplified due to competition. Some samples gave very faint bands for the *M. pulmonis* target sequences and brighter bands for the internal controls (see Fig. 1). Such results were interpreted as equivocal, although they were probably due to the presence of small amounts of the *M. pulmonis* target sequence, and were not tabulated as positive unless dot blotting and probing produced a distinct, unequivocal reaction.

Samples of reaction mixtures containing amplicons of the expected size were dot blotted onto positively charged nylon membranes and cross-linked with UV light. Membranes were probed (2 h at 65°C) with Mp.P1, a 37-base oligonucleotide that has the sequence 5′-CAAAG TGAAG CAAAC GCTCC TTTGA TATCT AAATC AT-3′ (positions 189 to 225) and that recognizes a sequence within each amplicon unique to *M. pulmonis*. The probe was labeled with digoxigenin (Oligonucleotide Tailing Kit; Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Denaturing solution, prehybridization buffer, hybridization buffer, and other reagents also were prepared according to the manufacturer's instructions. Blots with which the probe hybridized were visualized with alkaline phosphatase-conjugated anti-digoxigenin antibody and nitroblue tetrazolium as the chromogen (Genius 3 Nucleic Acid Detection Kit; Boehringer Mannheim).

Sensitivity was assessed by amplification of samples of serially 10-fold-diluted *M. pulmonis* DNA and logarithmic-phase cultures in medium A (4). Culture medium containing *M. pulmonis* was passed through a 0.4-μm-pore-size membrane filter before dilution, and both the numbers of CFU and the numbers of color-changing units were determined for each sample. To assess specificity, nested PCRs were done with samples of DNA from 18 strains of *M. pulmonis* and other mycoplasmas. *M. pulmonis* 5782C, 6510, 66, 7MCA1, 8145D, CT, Ginsburg, JB, M1, Negroni, Nelson A, Nelson C, Ogata T, Peter C, PG-22, PG-34, T4, and WRAIR were from stocks maintained in our laboratory from cultures originally isolated by us or obtained from the American Type Culture Collection (ATCC), the collection of J. G. Tully, or other laboratories. The nested PCR and probing of dot blots also were tested against nanogram quantities of DNA of *M. arthritidis*, *M. collis*, *Mycoplasma neurolyticum*, and *Mycoplasma fermentans*, which were obtained from ATCC and grown in SP-4 medium. Also tested were *Streptococcus pneumoniae*, *Corynebacterium kutscheri*, and *E. coli* (ATCC); DNA was extracted from broth cultures of each organism by standard chloroform-phenol extraction after washing three times by centrifugation and was quantified by spectrophotometry at 260 nm.

RESULTS

Amplicons that were of the expected sizes and that hybridized with the probe were obtained with DNAs of all 18 *M. pulmonis* strains, but not with those of *M. arthritidis*, *M. collis*, *M. neurolyticum*, *M. fermentans*, CAR bacillus, *S. pneumoniae*, *C. kutscheri*, or *E. coli*. The sensitivity of the first PCR, as determined from ethidium bromide-stained gels, was about 1,000 mycoplasma cells or the equivalent amount of DNA. The sensitivity after amplification in the second PCR, also as assessed from ethidium bromide-stained gels, was 1 to 10 mycoplasma cells. The sensitivities of both the first and second PCRs were similar whether they were expressed as the numbers of CFU or color-changing units or the amount of DNA. Dot blotting and probing did not substantially increase the sensitivity, and were therefore used to verify amplicon specificity rather than to increase sensitivity.

Results of nested PCR of 552 samples are summarized in Table 1, and an illustrative gel and dot blot are shown in Fig. 1 and 2, respectively. CAR bacillus samples were highly inhibitory, inasmuch as amplification was not detectable in the first PCR with 73 of 140 samples (52%), as indicated by the absence

TABLE 1. Nested PCR results for *M. pulmonis* for samples of all types

Sample	Nested PCR result (no. [%] of samples)						No. of samples tested
	Positive		Negative		Inhibitory		
	First PCR	Second PCR	First PCR	Second PCR	First PCR	Second PCR	
CAR bacillus	14 (10)	58 (41)	53 (38)	71 (51)	73 (52)	11 (7.9)	140
Mycoplasma culture	26 (79)	33 (100)	7 (21)	0	0	0	33
Lavage	1 (0.3)	25 (7.0)	323 (90)	319 (89)	35 (9.8)	15 (4.2)	359
Tissue section	0	14 (70)	17 (85)	6 (30)	3 (15)	0	20
Total	41 (7.4)	130 (24)	400 (72)	396 (72)	111 (20)	26 (4.7)	552

of amplicons of both *M. pulmonis* and the internal control. However, in the second or nested PCR, amplification failed with only 11 of 140 samples (7.9%), resulting in an additional 62 of 140 (44%) samples giving unequivocal results. Of these 62 samples, results were positive for 44 and negative for 18.

Table 2 presents the results of PCR of CAR bacillus samples by strain. In addition to the positive results obtained with samples of all six strains known from culture results to contain *M. pulmonis*, *M. pulmonis* was detected in two additional strains from which *M. pulmonis* could not be isolated. One of these, CAR bacillus X2006C, was isolated from a domestic rabbit; the other, CAR bacillus NIH, was derived from the original rat isolate (6). Of samples from mycoplasma broth cultures in which growth was suspected due to weak pH changes but from which mycoplasmas could not be subcultured on solid media, nested PCR increased the number of samples giving positive results in the first PCR from 26 of 33 (79%) to 33 of 33 (100%). Six of these samples were from cultures of CAR bacillus NIH, and 27 were cultures of respiratory tract lavage specimens from rats or mice inoculated with CAR bacillus NIH. None of the mycoplasma culture samples were inhibitory to either the first or the second PCR.

Inhibition was less prevalent with the other types of samples. Of 359 lavage specimens, only 35 (9.8%) were inhibitory in the first PCR and 15 (4.2%) were inhibitory in the second PCR. Nonetheless, the increased sensitivity and reduced prevalence of inhibition afforded by the second PCR resulted in an increase of 20 of 359 samples (5.6%) giving unequivocal results and an increase of 24 of 359 samples (6.7%) giving positive results. Sixty-eight lavage samples were from 72 rats inoculated with an avirulent strain of *M. pulmonis* (14). (Tracheobronchial lavage specimens were collected in addition to nasal lavage specimens if the nasal specimens contained blood.) The first PCR detected *M. pulmonis* in only one of two samples from 1 of 72 rats (1.4%), and positive culture results were obtained with specimens from only 2 of 72 rats (2.8%), whereas nested PCR gave positive results with all 21 samples from 14 of 72 rats (19%).

With fixed embedded tissues, nested PCR enabled detection of *M. pulmonis* in 14 of 20 (70%) of the samples, whereas in the first PCR no sample gave positive results and 3 of 20 (15%) samples were inhibitory. *M. pulmonis* was cultured from only 6 of 20 (30%) of the corresponding respiratory tract lavage samples. *M. pulmonis* was detected in sections from five of eight animals (63%) inoculated with CAR bacillus X1247C or X1331B, from which *M. pulmonis* had been isolated, and in 9 of 12 sections (75%) from animals inoculated with CAR bacillus NIH, from which *M. pulmonis* could not be cultured.

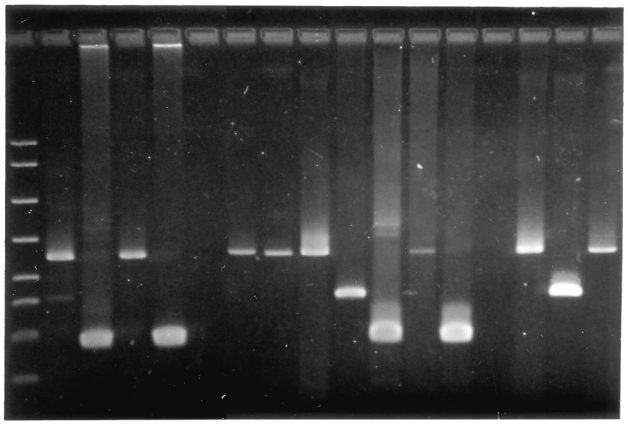

FIG. 1. Ethidium bromide-stained gel with products of nested PCR of samples of CAR bacillus NIH. Unnumbered lanes 1 to 17 are from left to right, respectively. Lane 1, ladder; lane 2, equivocal first PCR with distinct internal control band and very faint band at the expected position of the amplicon of the *M. pulmonis* target; lane 3, positive second PCR; lane 4, negative first PCR; lane 5, positive second PCR; lane 6, inhibitory sample in first PCR; lanes 7 to 9, negative first PCRs; lane 10, positive first PCR; lane 11, positive second PCR; lane 12, inhibitory first PCR with faint internal control band; lane 13, positive second PCR; lane 14, negative first PCR control; lane 15, first PCR control with internal control only; lane 16, positive first PCR control spiked with 10^4 copies of *M. pulmonis* DNA; lane 17, second PCR control with internal control only.

DISCUSSION

Nested PCR was clearly superior to single PCR for the detection of *M. pulmonis*. It was not only more sensitive, as expected, but was also less subject to inhibition, especially with CAR bacillus samples, which tended to be highly inhibitory. Nested PCR gave unequivocal results for 62 of 140 more CAR bacillus samples than single PCR, and of these 62 samples, results were positive for 44 and negative for only 18. Consequently, single PCR failed to detect *M. pulmonis* in a large fraction of contaminated CAR bacillus cultures, whereas nested PCR results showed that two CAR bacillus strains from which *M. pulmonis* could not be isolated (16) were contaminated with *M. pulmonis*. These results also demonstrate the value of internal controls for detecting inhibition of PCR. Single PCR with amplicon detection by ethidium bromide staining is reported to attain a sensitivity of about 1 pg of *M. pulmonis* DNA (7, 8, 10, 19). With a chromosome of about 950 kbp (12), this is the equivalent of about 1,000 *M. pulmonis*

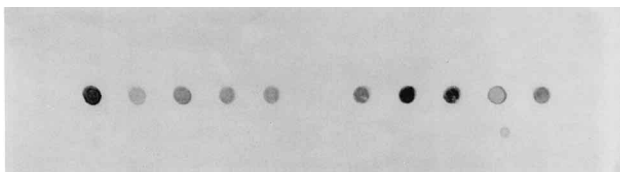

FIG. 2. Dot blots of PCRs with CAR bacillus NIH. Unnumbered wells 1 to 11 are from left to right, respectively. Well 1, *M. pulmonis*-positive control; wells 2 to 5, first PCRs interpreted as equivocal or weakly positive based on ethidium bromide stained gels; well 6, *E. coli* DNA control; wells 7 to 11, positive second PCRs.

TABLE 2. Results of nested PCR for *M. pulmonis* in CAR bacillus samples

M. pulmonis cultured[a]			*M. pulmonis* not cultured[b]		
Strain	First PCR result[c]	Second PCR result[c]	Strain	First PCR result[c]	Second PCR result[c]
StL	0/5	5/5	NIH	2/16	15/16
X1247C	1/6	6/6	X1328E	0/4	0/4
X1331B	5/6	6/6	X1328F	0/2	0/2
X1403B	0/2	1/2	X1328I	0/2	0/2
X1404B	0/2	1/2	X1428D/AS	0/5	0/5
X1428D	6/6	6/6	X2006C	0/6	6/6
			X2450D	0/7	0/7
			X2450E	0/7	0/7
			X2450F	0/7	0/7
			X2450G	0/5	0/5
Total	12/27 (44)	25/27 (93)		2/61 (3.3)	21/61 (34)

[a] CAR bacillus strains from which *M. pulmonis* was cultured.
[b] CAR bacillus strains from which *M. pulmonis* could not be cultured.
[c] Results are the number of samples giving positive results/number of samples tested (percent).

cells; thus, even moderate inhibition could prevent detection of substantial numbers of mycoplasmas.

We did not anticipate the degree of difficulty in identifying CAR bacillus cultures that do not contain *M. pulmonis*. Of the primary isolates tested, only those from rats from a single source (X2450*x*) did not contain *M. pulmonis. M. pulmonis* sequences were detected in several passages, up to the 10th passage in our laboratory, of the reference CAR bacillus strain (strain NIH). Lavage specimens, mycoplasma cultures, and tissue sections from animals inoculated with CAR bacillus NIH up to 12 weeks previously also gave positive nested PCR results. In addition, we previously found by transmission electron microscopy that samples of this strain contained bodies consistent with mycoplasmas (16). Therefore, we conclude that this strain contains viable *M. pulmonis* organisms and that it was probably present in the culture that we originally received. Samples of the rabbit isolate (X2006C) also had previously been found to contain mycoplasma-like bodies by electron microscopy (16), and samples from passages up to the sixth passage gave positive nested PCR results. Thus, we conclude that this strain also contains viable *M. pulmonis* organisms. This is not altogether unexpected, inasmuch as *M. pulmonis* has been isolated from rabbits (1).

Nested PCR also was superior to single PCR for the detection of *M. pulmonis* in respiratory tract lavage samples and sections of formalin-fixed paraffin-embedded tissues. The procedure also detected *M. pulmonis* sequences in mycoplasma broth cultures in which growth could not be confirmed by subculturing, in addition to CAR bacillus cultures from which *M. pulmonis* could not be cultured. This shows that although *M. pulmonis* usually is rather readily cultured in comparison with some other mycoplasmas, strains of *M. pulmonis* that are noncultivable or at least very difficult to cultivate are occasionally encountered. Infection with such a strain could affect the results of rodent health assessments. Such assessments are necessary to ensure that research in which rats are used is not compromised by infectious agents that could alter the results. *M. pulmonis* is one of the most important of such agents, although it is far less prevalent now than it was in the past (11, 15). Nested PCR could provide more definitive results than current methods both in rodent health assessments and in the diagnosis of naturally occurring respiratory disease. However, nested PCR is technically demanding. False-positive results are a particular problem and can occur even with diligent efforts to prevent sample contamination and product carryover. Nested PCR also is time-consuming and would have to be adapted to automation to be practical for rodent health screening.

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