Detection of *Mycoplasma pulmonis* in Experimentally Infected Laboratory Rats by 16S rRNA Amplification

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Recently, an rRNA-based polymerase chain reaction (PCR) has been developed for the detection of murine mycoplasmas at both the genus and species level (F. J. M. van Kuppeveld, J. T. M. van der Logt, A. F. Angulo, M. J. van Zoest, W. G. V. Quint, H. G. Niesters, J. M. D. Galama, and W. J. G. Melchers, Appl. Environ. Microbiol. 58:2606–2615, 1992). In this study, the diagnostic value of this PCR assay for the detection of *Mycoplasma pulmonis* in infected rats was studied. For this purpose, 25 Wistar rats were infected intranasally with *M. pulmonis* strain M72-138 and investigated for the presence of this pathogen by both in vitro isolation and PCR. Five rats were monitored longitudinally by screening of throat swabs at several time points for up to 248 days postinfection. The remaining 20 rats were killed between 3 and 87 days postinfection, and organism recovery from both throat and urogenital tract specimens was attempted. *M. pulmonis* could be detected in the throat for up to 248 days postinfection but not in the urogenital tract, either by culture or by PCR. PCR proved to be the optimal method for testing throat samples. All samples in which *M. pulmonis* was detected by culture were also positive by PCR. By PCR, *M. pulmonis* was also detected in 3.7% of the samples which were culture negative and in 9.9% of the samples from which cultures were overgrown with bacteria. The results of this study demonstrate the suitability of PCR for the detection of mycoplasmal infection in rodents.

Mycoplasmal infections may cause severe problems in laboratory rodent colonies. Recent surveys indicate that about 60% of barrier-maintained and nearly all conventionally housed laboratory rat and mouse colonies are infected with one or more mycoplasmas (8, 10). The mycoplasmal species isolated from rats and mice (*Mycoplasma pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. muris*, and *M. collis*) have been associated with several disease manifestations. One of the main diseases is murine respiratory mycoplasmosis, or chronic respiratory disease, which is caused by *M. pulmonis* and involves the nasal passages, middle ears, trachea, and lungs, causing rhinitis, otitis media, tracheitis, and pneumonia (5, 11). Besides respiratory disease, this organism can also produce genital infections, resulting in reduced birth rates (1, 5, 6). However, in spite of their pathogenic potential, mycoplasmal infections can remain inapparent. These inapparent infections may be very treacherous because mycoplasmas possess immunomodulatory activities which can influence the outcome of experiments (4, 5).

A first step in building and maintaining a mycoplasma-free colony is the use of an efficient detection system to routinely identify mycoplasma carriers (3). The methods currently used for the diagnosis of mycoplasmal infections show several shortcomings. In vitro isolation, which requires multisite culture for reliable results (3), is laborious and time-consuming and lacks sensitivity for several species, while serological methods are often hampered by cross-reactions between different species (3, 4, 12). In addition, it has also been shown that animals can be infected and remain seronegative for weeks after infection (2).

Recently, we developed a polymerase chain reaction (PCR) assay for the detection of mycoplasmas at both the

species and genus level (13). Primers selected from the variable 16S rRNA regions of the five murine mycoplasmal species were all species specific in this PCR assay. With a high copy number of rRNA (10^4 copies per cell), a highly sensitive assay was established in which the nucleic acid content equivalent to a single microorganism could be detected (13).

In this study, the suitability of this PCR assay for the detection of mycoplasmal infection was tested in Wistar rats that were experimentally infected with *M. pulmonis*. We compared culture and the PCR assay for the detection of *M. pulmonis* in throat and urogenital tract samples collected at several times postinfection.

MATERIALS AND METHODS

Animals. Twenty-nine germ-free Cpb:WU (Wistar) rats, male and female, aged 10 weeks, were used. These rats are proven to be serologically negative for *M. pulmonis*. The rats were kept in sterile isolators and fed sterilized rat-mice food. Room temperature and humidity were regulated (21 ± 2°C, 60% ± 10% relative humidity). The rats were housed in Macrolon cages, three animals per cage, with softwood granules as bedding. Noninfected control rats were housed separately from the experimentally infected rats.

Media. For cultivation in liquid and agar media, a modification of the medium described by Chanock et al. (7) was used. The liquid culture medium contained (in milliliters per 100 ml): 2.1% Bacto P.P.L.O. Broth (Difco), 70; 25% yeast extract (Difco), 10; 10% glucose (Merck), 10; inactivated gamma globulin-free horse serum (GIBCO), 5; P.P.L.O. serum fraction (Difco), 2; 0.1% phenol red (Sigma), 1; 2.5% thallium acetate (Merck), 1.4; and sodium penicillin G (10^5 U/ml) (Gist-Brocades), 1. The pH was adjusted to 8.0 with 1 N KOH.

Mycoplasma agar plates contained (in milliliters per 100

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ml): 3.4% Bacto P.P.L.O. Agar (Difco), 70; inactivated gamma globulin-free horse serum, 20; 25% yeast extract, 10; 10% glucose, 10; 0.1% phenol red, 4; and 2.5% thallium acetate, 2. The pH was adjusted to 8.0 with KOH.

Experimental inoculation of animals. The *M. pulmonis* strain used for this study (M72-138) was originally isolated from the trachea of a rat from an infected laboratory colony. This strain was passaged three times on artificial medium. For this experiment, the mycoplasma was grown in liquid culture medium and harvested when the medium turned orange-red. The number of CFU was determined by titration on agar plates. The inoculum was kept at −80°C until use.

Twenty-five rats (rats 1 through 25) were experimentally infected with 10^6 CFU of *M. pulmonis* (in 100 μl of culture medium) by intranasal inoculation. Four noninfected rats (rats 26 through 29), housed separately from the infected rats, were used as controls.

Evaluation of infection and collection of samples. At 14 time points (3, 7, 10, 14, 21, 31, 42, 59, 73, 87, 117, 151, 206, and 248 days postinfection), throat swab samples were taken from rats 1 through 5. After 248 days, these rats were killed by cervical dislocation and urogenital swab samples (vaginal for female rats and urethral for male rats) were collected. Both throat and urogenital swab samples were collected at necropsy from rats 6 through 25, two of which were killed at each of the first 10 time points (3 to 87 days postinfection). Throat and urogenital swab samples were also taken from control rats 26 through 29, one of which was killed on each of days 7, 14, 21, and 59 postinfection.

The throat and urogenital swab samples were collected with sterile Dacron-tipped swabs (swab MW142; Medical Wire & Equipment Co. Ltd.). The swabs were suspended in 2 ml of liquid culture medium. Half of the suspended swabs were used for culture, and the other half were placed at −80°C for PCR analysis.

PCR analysis. Samples (1 ml) obtained from the throat and urogenital swabs were centrifuged for 10 min at 10,000 × *g*. RNA was extracted by the RNAzol B method (Cinna Biotecx) according to the manufacturer's instructions. cDNA synthesis of the RNA, the PCR assay, analysis of the amplified samples by electrophoresis, and subsequent hybridization were performed as described previously (13). PCR was performed with *M. pulmonis* species-specific primers (sense primer, 5'-AGCGTTTGCTTCACTTTGAA-3'; antisense primer, 5'-GGGCATTTCCTCCCTAAGCT-3'), which generate a 266-bp amplification product. The thermal profile involved 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 2 min. Hybridization was performed with the internal ^{32}P-end-labeled oligonucleotide GPO-1 (5'-ACTCCT ACGGGAGGCAGCAGTA-3').

Culture. The suspended throat and urogenital swab samples were incubated at 37°C until the color of the medium changed to orange-red. Cultures were incubated for 6 weeks before being considered negative.

Positive cultures were identified as *M. pulmonis* by PCR, since we had previously demonstrated (unpublished data) a 100% correlation between identification of the organisms grown in culture medium by the growth inhibition test and identification by PCR with species-specific primers. For this PCR, DNA was extracted from the organisms grown in culture medium, and rDNA sequences were amplified as described previously (13), with the *M. pulmonis*-specific primers described above. *M. pulmonis* was not demonstrated in any of the cultures which did not display a change of color.

TABLE 1. Detection of *M. pulmonis* by culture and PCR analysis[a]

Day p.i.	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5	
	C	P	C	P	C	P	C	P	C	P
3	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+
31	BC	+	+	+	BC	+	+	+	BC	+
42	+	+	+	+	−	−	+	+	+	+
59	+	+	+	+	−	−	+	+	+	+
73	+	+	+	+	−	−	BC	+	+	+
87	+	+	+	+	−	−	+	+	+	+
117	+	+	+	+	−	−	+	+	+	+
151	−	−	+	+	−	−	+	+	+	+
206	+	+	+	+	−	−	+	+	+	+
248	−	+	−	+	−	−	+	+	+	+

[a] Throat swab samples were collected from rats 1 through 5 on the indicated days postinfection (p.i.) and tested for *M. pulmonis* by culture (C) and PCR (P).

[b] BC, bacterial contamination.

ELISA. Routine enzyme-linked immunosorbent assays (ELISAs) for immunoglobulin G antibodies to *M. pulmonis* were performed as described by Cassell et al. (3), with serum samples collected from rats 1 through 5 at the first 10 time points and from rats 6 through 29 on the day of death.

RESULTS

Culture and PCR analysis were compared for the detection of *M. pulmonis* in the throat and urogenital tract of 25 experimentally infected Wistar rats. Infection of these rats was confirmed by ELISA for the detection of immunoglobulin G antibodies against *M. pulmonis* (data not shown).

Rats 1 through 5 were investigated longitudinally for the presence of *M. pulmonis* in the throat at several time points between 3 and 248 days postinfection. Table 1 demonstrates that with the PCR assay, which includes Southern blot analysis, *M. pulmonis* was detected in the throat of rats 1, 2, 4, and 5 at all time points investigated, while culture failed to detect *M. pulmonis* in two samples (throat swabs from rats 1 and 2 collected 248 days postinfection). The failure to detect *M. pulmonis* in the throat of rat 1 at day 151 postinfection by both PCR and culture is probably the result of an error in collecting the sample, since subsequent samples were again positive for *M. pulmonis*. Remarkably, *M. pulmonis* was no longer detected in the throat of rat 3 from day 42 postinfection.

Rats 6 through 25 and control rats 26 through 29 were killed at various times postinfection and investigated for the presence of *M. pulmonis* in both the throat and urogenital tract. The culture and PCR results, which are shown in Table 2, demonstrate that *M. pulmonis* was detected in the throat of all infected rats but not in the control rats by PCR, whereas with the culture technique, the organism was missed in one sample (throat swab from rat 7, collected 3 days postinfection). Table 2 also demonstrates that in none of the infected rats was *M. pulmonis* detected in the urogenital tract. Also, *M. pulmonis* was not detected in the urogenital tract of rats 1 through 5, which were killed 248 days postinfection (data not shown).

TABLE 2. Detection of *M. pulmonis* by culture and PCR in throats and urogenital tracts of experimentally infected and noninfected control rats[a]

Rat group	No. of positive samples/no. tested			
	Throat		Urogenital tract	
	Culture	PCR	Culture	PCR
Infected	15/16[b]	20/20	0/20	0/20
Control	0/4	0/4	0/4	0/4

[a] Infected rats 6 through 25 and control rats 26 through 29 were killed at various times postinfection (see Materials and Methods), and swab samples were collected from the throat and urogenital tract at necropsy.

[b] Bacterial overgrowth was observed in four cases.

Figure 1 shows an example of an electrophoretic analysis of the PCR products. Besides the 266-bp fragment, a smaller fragment (about 175 bp) was amplified in the RNA PCR which was not found in the DNA PCR. This 175-bp fragment also hybridized with the internal probe (data not shown). The origin of this smaller product is unknown. The addition of Perfect Match Enhancer (Stratagene), which destabilizes mismatched primer-template complexes, or dactinomycin, which reduces secondary RNA structures, did not prevent the amplification of this smaller product (data not shown).

DISCUSSION

In this study, the suitability of the 16S rRNA-based mycoplasmal PCR assay (13) as a tool for the diagnosis of mycoplasmal infection was examined in Wistar rats experimentally infected with *M. pulmonis*. In a preliminary study (data not shown), we had found that *M. pulmonis* could be detected in throat swab samples of experimentally infected rats by PCR analysis. It was also found that the sensitivity was increased when the rRNA instead of the rDNA target was used, since the rDNA PCR failed to detect *M. pulmonis* in some samples which were positive in the rRNA PCR. Therefore, in the current study, the rRNA PCR was performed for the detection of *M. pulmonis*.

Throat and urogenital tract swab samples were collected from 25 experimentally infected rats at several time points postinfection and tested by both PCR and in vitro isolation. The results demonstrate that *M. pulmonis* was detected in the throat of all infected rats by PCR. By culture, *M. pulmonis* was detected in only 70 (86.4%) of the 81 samples

that were positive in the PCR. Bacterial overgrowth was observed in eight samples (9.9%), and *M. pulmonis* was not detected by the culture method in three samples.

Detection of this mycoplasma in the respiratory tract for at least 248 days postinfection is consistent with the findings of Lindsey et al. (11), who reported that *M. pulmonis* persisted in mice for several months. Why rat 3 was no longer positive for *M. pulmonis* after 31 days postinfection is unknown. In contrast to the throat samples, the urogenital tract samples of all rats were negative. It has been reported that it is extremely difficult to recover mycoplasmas from the genital tracts of male rats (9). However, since all the urogenital samples from female rats were also negative for *M. pulmonis*, it is most probable that the *M. pulmonis* strain used in this experiment has not colonized the urogenital tract of these Wistar rats.

In summary, the results of this study indicate that PCR is superior to culture for the detection of *M. pulmonis* in infected rats. These results therefore demonstrate the applicability of this PCR assay for the detection of mycoplasmas in rodents. Currently, the diagnosis of mycoplasmal infections is performed by ELISA and/or in vitro isolation. In vitro isolation of mycoplasmas, which includes cultivation and identification, is laborious, time-consuming, and subject to bacterial contamination and lacks sensitivity for several mycoplasmal species. PCR, however, is a rapid, specific, and sensitive procedure. Therefore, PCR may replace in vitro isolation and become the method of choice in multisite screening programs to identify mycoplasma carriers. We are currently using this PCR assay to screen laboratory rodent colonies for naturally infected animals.
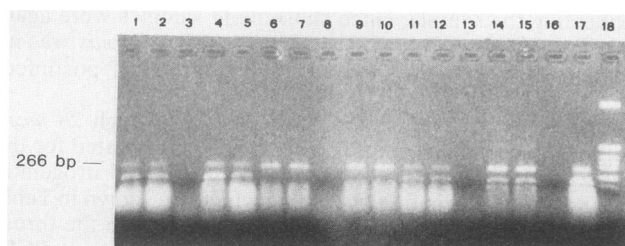

FIG. 1. Agarose gel electrophoretic analysis of the PCR assay performed with RNA extracted from throat swab samples of rat 1 (lanes 1, 6, and 11), rat 2 (lanes 2, 7, and 12), rat 3 (lanes 3, 8, and 13), rat 4 (lanes 4, 9, and 14), and rat 5 (lanes 5, 10, and 15) collected at 73 days (lanes 1 to 5), 87 days (lanes 6 to 10), and 117 days (lanes 11 to 15) postinfection. Lane 16 and 17 show a negative and a positive (*M. pulmonis* RNA) control, respectively. Lane 18 shows size markers (pBR322 digested with *Hin*fI).

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