JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1995, p. 893–897 Vol. 33, No. 4
0095-1137/95/$04.00+0

Detection of *Mycoplasma hyopneumoniae* in Nose Swabs from Pigs by In Vitro Amplification of the 16S rRNA Gene

JENS G. MATTSSON, KATRIN BERGSTRÖM, PER WALLGREN, AND KARL-ERIK JOHANSSON*

National Veterinary Institute, S-750 07 Uppsala, Sweden

Received 11 July 1994/Returned for modification 10 August 1994/Accepted 19 January 1995

We report the use of PCR to detect DNA from *Mycoplasma hyopneumoniae*, the etiological agent of enzootic porcine pneumonia. A primer set was designed for the amplification of a 649-bp fragment of the 16S rRNA gene from *M. hyopneumoniae*. The PCR product was identified by ethidium bromide staining after gel electrophoresis and by Southern hybridization with an *M. hyopneumoniae*-specific oligonucleotide probe. No amplification was observed from any other porcine bacteria tested, including several closely related mycoplasmas. It was also possible to demonstrate the presence of *M. hyopneumoniae* in bronchial lavage samples and lung tissue samples from experimentally infected pigs. Furthermore, the PCR system was used for analysis of nasal samples obtained from pigs in a fattening herd. By this method, we were able to detect *M. hyopneumoniae* in nose swabs from naturally infected pigs. However, our results suggest that *M. hyopneumoniae* can be detected in the nasal cavities only during a limited time period.

Members of the cell wall-deficient class *Mollicutes* are the smallest self-replicating organisms known (12). They possess the smallest genome of any free-living organism, and the G+C content of their DNA is among the lowest known. The class consists of eight different genera, of which the genus *Mycoplasma* is the most well known (25). The different mycoplasmas are more or less host specific, and some of them are significant animal pathogens. In 1965 *Mycoplasma hyopneumoniae* was recognized as the causative agent of enzootic pneumonia in swine (EP) (7, 13). The disease has been reported from many countries and is one of the most common and economically serious diseases occurring in swine (18). However, isolation of the organism is complicated by its fastidious nature and extremely slow growth. It may be necessary to incubate primary broth cultures for 30 days after inoculation. After several passages in broth medium, cultures can be inoculated on agar medium for identification by immunofluorescence methods. During the isolation, *M. hyopneumoniae* is easily overgrown by other mycoplasmas; *Mycoplasma hyorhinis* is, for instance, frequently found in swine suffering from EP. Furthermore, the nonpathogenic *Mycoplasma flocculare* is commonly found in swine lungs and has many morphologic, growth, and antigenic characteristics in common with *M. hyopneumoniae* (3, 18). These similarities are also supported by molecular data (10, 21, 23).

It is often not feasible to diagnose EP by cultivation of *M. hyopneumoniae*. So far, the organism has been successfully recovered only from slaughtered pigs, normally from lung tissues (18). The probable route of transmission is by direct contact with respiratory tract secretions from infected swine, and *M. hyopneumoniae* has been isolated from nasal cavities of diseased pigs (6). With the introduction of PCR (20) it is now possible to track down microorganisms that were very difficult to detect by classical methods. A PCR system for the detection of a reiterative sequence in genomic DNA from *M. hyopneumoniae* has been described previously (9), and more recently a PCR system with primers complementary to an unknown genomic fragment has also been tested (1). In addition, a PCR system with primers complementary to the 16S rRNA gene has also been described (22). However, none of these systems were used to analyze clinical samples. We have earlier reported about an oligonucleotide probe targeting 16S rRNA suitable for direct detection of *M. hyopneumoniae* in lung biopsy specimens from pigs suffering from pneumonia in the acute phase (10). In the present work we describe a PCR system based on amplification of the 16S rRNA gene for the detection of *M. hyopneumoniae* and the testing of this PCR on nasal swabs taken from conventional pigs in a fattening herd.

* Corresponding author. Mailing address: National Veterinary Institute, P.O. Box 7073, S-750 07 Uppsala, Sweden. Phone: 46 18 67 40 00. Fax: 46 18 30 91 62. Electronic mail address: Karl-Erik.Johansson@vetmed.sva.se(Internet).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The mycoplasmas and other bacteria used in this work are listed in Table 1. Most of the mycoplasmas were obtained from the former WHO/FAO Collaborating Centre for Animal Mycoplasmas (Aarhus, Denmark). The exceptions were the type strains of *M. hyopneumoniae* and *M. hyorhinis*, which were obtained from the National Collection of Type Cultures (London, United Kingdom); *Mycoplasma hyopharyngis*, which was obtained from R. F. Ross (Iowa State University); and the Ms strains of *M. hyopneumoniae*, which were obtained from N. Friis (Aarhus University). The M-labeled isolates of *M. hyopneumoniae* and all other bacteria were from the culture collection of our institute, except for *Erysipelothrix rhusiopathiae*, which was obtained from the American Type Culture Collection (Rockville, Md).

The porcine mycoplasmas *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* were cultivated in the medium described by Friis (5). The other mycoplasmas were grown in F medium or HA medium (2). All mycoplasmas were checked by immunofluorescence, and the amount of mycoplasmas used in the PCR experiments was estimated by counting CFU as described elsewhere (16).

In vitro amplification by PCR. One milliliter of broth culture of each bacterium tested by PCR was centrifuged, washed in phosphate-buffered saline (PBS), resuspended in water, and lysed by heating at 100°C for 5 min. DNA from 5-μl samples of undiluted and 10-fold-diluted lysed cells was used as PCR templates. A 649-bp fragment of the 16S rRNA gene from *M. hyopneumoniae* was amplified by PCR with the forward primer 5′-GAG CCT TCA AGC TTC ACC AAG A-3′ (nucleotide positions 212 to 233 in the *M. hyopneumoniae* 16S rRNA sequence) and the reverse primer 5′-TGT GTT AGT GAC TTT TGC CAC C-3′ (nucleotide positions 839 to 860). The 16S rRNA sequence of *M. hyopneumoniae* was obtained from GenBank (accession number M30378). The amplification was performed in a 50-μl reaction mixture containing a 0.2 mM concentration of each deoxynucleotide, 20 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl buffer (pH 9.0), and 2 mM $MgCl_2$. Two droplets of mineral oil were layered on the reaction mixtures to prevent evaporation. The reaction mixtures were incubated at 95°C for 4 min and then transferred to a separate heating block at 82°C, where 1 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added (17). The thermal profile involved 35 cycles of denaturation at 95°C for 45 s, primer annealing at 60°C for 1 min, and extension at 72°C for 2 min. The

TABLE 1. Bacterial strains analyzed by the *M. hyopneumoniae*-specific PCR

Species	Strain[a]	PCR result[b]
Mycoplasma hyopneumoniae	J^T	+
Mycoplasma hyopneumoniae	Ms41	+
Mycoplasma hyopneumoniae	Ms43	+
Mycoplasma hyopneumoniae	Ms44	+
Mycoplasma hyopneumoniae	Ms53	+
Mycoplasma hyopneumoniae	Ms58	+
Mycoplasma hyopneumoniae	M13/79	+
Mycoplasma hyopneumoniae	M105/79	+
Mycoplasma hyopneumoniae	M158/86	+
Mycoplasma hyopneumoniae	M95/87	+
Mycoplasma hyopneumoniae	M62/88	+
Mycoplasma arginini	$G230^T$	−
Mycoplasma flocculare	$Ms42^T$	−
Mycoplasma hyorhinis	$BTS\text{-}7^T$	−
Mycoplasma hyopharyngis	538N	−
Mycoplasma hyosynoviae	$S\ 16^T$	−
Mycoplasma sualvi	Mayfield B^T	−
Acholeplasma axanthum	$S743^T$	−
Acholeplasma granularum	$BTS\text{-}39^T$	−
Acholeplasma laidlawii	$PG8^T$	−
Pasteurella multocida	2457	−
Haemophilus parasuis	B26	−
Corynebacterium pyogenes	B429/83	−
Actinobacillus pleuropneumoniae	Bd 4611	−
Staphylococcus aureus	Oxford 209	−
Erysipelothrix rhusiopathiae	ATCC 19414^T	−
Bordetella bronchiseptica	B933	−

[a] T, type strain; ATCC, American Type Culture Collection.
[b] +, positive; −, negative.

amplifications were performed in a model 480 thermocycler (Perkin-Elmer Cetus).

Analysis of the amplified samples. Aliquots of amplified samples (10 μl) were analyzed by electrophoresis in a 1.5% agarose gel with 0.5 μg of ethidium bromide per ml. An alkaline transfer method was used for Southern blotting. Briefly, no pretreatment of the gel was performed prior to the transfer; instead, 0.4 M NaOH was used to denature the DNA during the transfer.

After fixation, the membrane (Zeta Probe GT; Bio-Rad Laboratories, Richmond, Calif.) was prehybridized for 30 min at 65°C in a mixture of 2× SSC (1× SSC is 150 mM NaCl–15 mM sodium citrate), 10× Denhardt's solution (50× Denhardt's solution is 1% bovine serum albumin–1% polyvinylpyrrolidone–1% Ficoll), 2% sodium dodecyl sulfate, and denatured herring sperm DNA (150 μg/ml). The *M. hyopneumoniae*-specific oligonucleotide Mhp 6/30 (5′-CCG TCA AGA CTA GAG CAT TTC CTA TCT AGT-3′) complementary to the V6 region of 16S rRNA (10) was end labeled with [γ-^{32}P]ATP (Amersham, Little Chalfont, United Kingdom) and T4 polynucleotide kinase (Pharmacia Biotech, Uppsala, Sweden). V is used to designate variable nonconserved regions in 16S rRNA as defined by Gray et al. (8). About 10^6 cpm of the probe per ml was added to the prehybridization solution. After 2 h of hybridization at 65°C, the membrane was washed with 2× SSC, once at 65°C for 5 min and three times at 55°C for 10 min, before autoradiography.

Samples from experimentally infected pigs. Bronchial lavage samples and ground lung tissue samples from experimentally infected pigs were prepared as described earlier (10). Prior to the PCR, 100-μl samples were washed in PBS, resuspended in water, and lysed by boiling as described above. Five-microliter aliquots of undiluted and 10-fold-diluted samples were used as PCR templates. Bronchial lavage samples and lung tissue samples from barrier-maintained pigs were also analyzed by PCR.

Clinical samples. To evaluate the PCR system under field conditions, nasal swabs and blood samples were collected from pigs in a conventional fattening herd with 510 stalls, in an all in-all out production system. Thirty-one pigs, evenly distributed in the stable, were sampled 12 weeks after arrival to the herd. Pigs not yet slaughtered ($n = 27$) were resampled 3 weeks later. The nasal swabs were transported in 50 μl of PBS and stored frozen at −70°C until further use. The swabs were vortexed and discarded, and the PBS was heated at 100°C for 5 min before PCR. Five microliters of undiluted and 10-fold-diluted samples was used in PCR. Blood samples (without additives) were centrifuged at 2,000 × *g* for 10 min, and the sera were stored at −20°C until analyzed for presence of antibodies to *M. hyopneumoniae* by an enzyme-linked immunosorbent assay (ELISA) previously described (28).

RESULTS

Design of PCR primers. PCR primers were chosen on the basis of published 16S rRNA sequence data from porcine and other mycoplasmas (10, 21, 24, 31). The forward primer was complementary to a part of the V2 region, and the reverse primer was complementary to a subdomain of the V8 region.

Specificity of the PCR. A 649-bp PCR product was observed for *M. hyopneumoniae* (Fig. 1). A total of 10 field strains were examined by PCR. All of them reacted positively (Table 1). All of the other mycoplasmas and bacteria tested in the PCR were negative. All tested bacteria have swine as their only or alternative host. The results from these experiments are summarized in Table 1. However, at lower annealing temperatures (≤56°C) a fragment larger than 0.9 kb was generated with DNA from *Pasteurella multocida*. This suggested that it was not a part of the 16S rRNA gene that was amplified. The Mhp 6/30 probe hybridized only with the PCR product from *M. hyopneumoniae.*

Sensitivity of the PCR. The amount of mycoplasmas from the broth culture of *M. hyopneumoniae* was determined by CFU. Serial 10-fold dilutions of *M. hyopneumoniae* cells were analyzed by PCR. Amplified DNA from cells corresponding to 5 CFU could be detected by agarose gel electrophoresis (data not shown). This sensitivity was achieved in several independent dilution series. The use of the Mhp 6/30 probe for hybridization with PCR products enhanced the signal strength compared with that by direct visualization with ethidium bromide. However, 5 CFU appeared to be the threshold for the PCR.

Experimentally infected pigs. Lung tissue samples and bron-

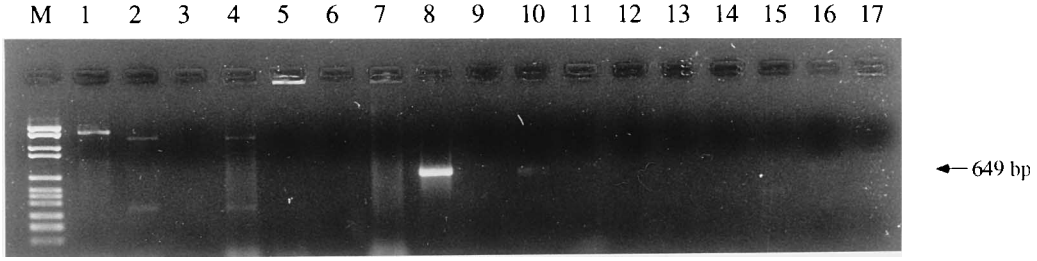

FIG. 1. Agarose gel electrophoresis of the material obtained by PCR experiments with the strains listed in Table 1. Lane 1, *P. multocida*; lane 2, *Haemophilus parasuis*; lane 3, *Corynebacterium pyogenes*; lane 4, *Actinobacillus pleuropneumoniae*; lane 5, *Staphylococcus aureus*; lane 6, *E. rhusiopathiae*; lane 7, *Bordetella bronchiseptica*; lane 8, *M. hyopneumoniae*; lane 9, *Mycoplasma arginini*; lane 10, *M. flocculare*; lane 11, *Mycoplasma sualvi*; lane 12, *M. hyorhinis*; lane 13, *M. hyopharyngis*; lane 14, *M. hyosynoviae*; lane 15, *Acholeplasma laidlawii*; lane 16, *Acholeplasma granularum*; lane 17, *Acholeplasma axanthum*. *Bgl*I-cleaved pBR 328 DNA and *Hin*fI-cleaved pBR 328 DNA from Boehringer Mannheim (Mannheim, Germany) were used as molecular-size markers in lane M.

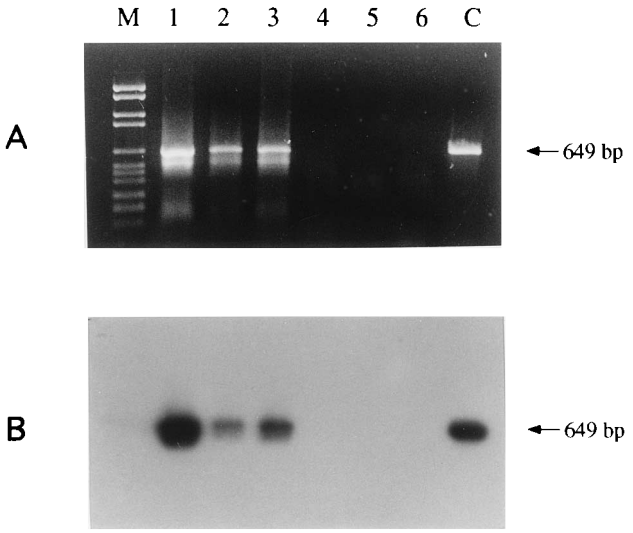

FIG. 2. Agarose gel electrophoresis of the material obtained by PCR experiments (A) and after Southern blot analysis of the gel shown in panel A (B). The samples were from three pigs experimentally infected with *M. hyopneumoniae* (lanes 1 to 3), two uninfected barrier-maintained pigs (lanes 4 and 5), one conventional fattening pig aged 1 week (lanes 6), and a positive control (lanes C). A bronchial lavage sample was analyzed in lanes 2, and in the rest of the lanes lung tissue samples were analyzed by the PCR. The markers in lanes M were identical to those used in Fig. 1.

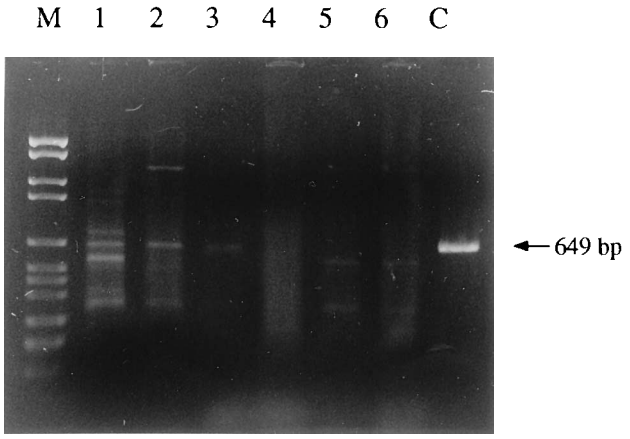

FIG. 3. Agarose gel electrophoresis of the material obtained by PCR experiments from six swine in a fattening herd (lanes 1 to 6) and a positive control (lane C). PCR products identical in size as compared with the positive control were obtained in lanes 1 and 2. These positive samples were also confirmed by Southern blot analysis (data not shown). The markers in lane M were identical to those used in Fig. 1.

chial lavage samples from three pigs experimentally infected with *M. hyopneumoniae* were analyzed by PCR. The result from the PCR is shown in Fig. 2A. All samples were positive in one or two of the dilutions tested. *M. hyopneumoniae* was cultivated from the lung tissue samples but not from the bronchial lavage samples. No amplification was observed with the samples from the barrier-born pigs and from lung tissue samples from a conventional pig, aged 1 week. However, *M. hyorhinis* was cultivated from the latter pig.

Clinical samples. At the first sampling occasion (12 weeks after arrival to the fattening herd), *M. hyopneumoniae* was demonstrated in five of the pigs, both by PCR and after hybridization. In Fig. 3, samples from six of the pigs are shown. Several unspecific PCR products were observed after gel electrophoresis. Serum antibodies to *M. hyopneumoniae* were demonstrated by ELISA in 24 of the pigs 12 weeks after the arrival. One of the pigs in which *M. hyopneumoniae* was detected by PCR did not express serum antibodies reactive to *M. hyopneumoniae* at this time. However, this pig was seropositive for *M. hyopneumoniae* in ELISA but negative by PCR 3 weeks later. Of the other four pigs in which *M. hyopneumoniae* was shown by PCR at the first sampling occasion, in only one was *M. hyopneumoniae* found 3 weeks later. At the latter sampling, *M. hyopneumoniae* was demonstrated in one of the pigs negative for *M. hyopneumoniae* 12 weeks after arrival. Fifteen weeks after arrival, all but one animal had serum antibodies to *M. hyopneumoniae*. This animal was also negative by PCR at both samplings (Fig. 4).

DISCUSSION

Since the mid-1970s there has been a continuously growing interest in the possibilities in using rRNA sequences to infer phylogenetic interrelationships among microorganisms (15, 32, 33). During the last several years an increasing number of applications have also been developed for the diagnosis and detection of bacteria with probes or PCR primers targeting 16S rRNA or the corresponding genes (14, 29). Several of the characteristics of the 16S rRNA sequences utilized for the calculation of phylogenetic trees are valuable also for the design of PCR primers. The mosaic organization of 16S rRNA with highly conserved regions interspersed with regions of moderate and high sequence variability allows for comparisons on a wide spectrum of phylogenetic distances (15). A PCR system can in a similar manner be used to amplify 16S rRNA genes on different phylogenetic levels, ranging from species and groups to higher levels such as genera and bacterial phyla (4, 11, 17, 30).

In this work we have described a PCR system for the detection of *M. hyopneumoniae*, the etiological agent of EP. The primers were complementary to evolutionarily variable regions of the 16S rRNA gene from *M. hyopneumoniae*. A similar system has been described by Stemke et al. with primers com-

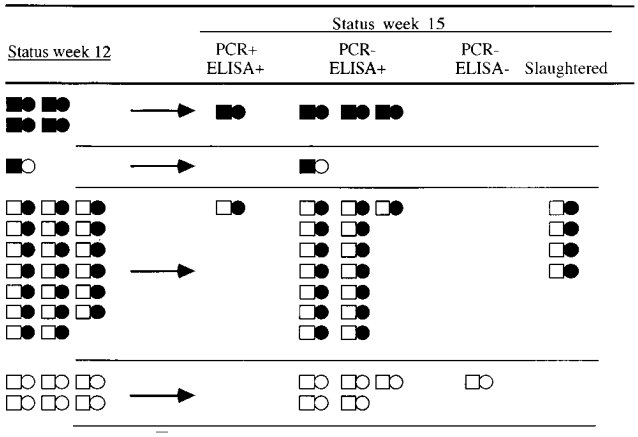

FIG. 4. Evidence of infection with *M. hyopneumoniae* in conventional fattening pigs 12 and 15 weeks after arrival. *M. hyopneumoniae* was demonstrated in nasal swabs by PCR or reflected as the presence of serum antibodies. The symbols represent the status of the animals 12 weeks after arrival to the herd: ■●, PCR positive, ELISA positive; ■○, PCR positive, ELISA negative; □●, PCR negative, ELISA positive; □○, PCR negative, ELISA negative.

plementary to the 16S rRNA gene (22). However, their PCR was performed only on pure DNA, and it was not tested on porcine bacteria other than mycoplasmas; nor were any clinical samples included. To increase the specificity and sensitivity in our assay, a hybridization step with a probe was used. The probe was complementary to a part of the PCR product derived from the V6 region of the 16S rRNA of *M. hyopneumoniae* (10).

The organism could readily be detected in samples from lung tissues and bronchial lavage samples from experimentally infected pigs. Normally 90% of the pigs from fattening herds in Sweden have serum antibodies to *M. hyopneumoniae* at slaughter (26). Careful evaluation of lungs at slaughter suggests that most of the animals also have experienced pneumonia, even though the lesion might be in a healing phase at the time of slaughter (27). We detected a similar prevalence of pigs with antibodies to *M. hyopneumoniae* in this study despite the fact that *M. hyopneumoniae* was detected in only 16 and 4% of the clinical samples tested by PCR 12 and 15 weeks after arrival, respectively. There may be several plausible explanations for this discrepancy. An excess of cellular debris can inhibit PCR, and a more extensive DNA purification may have to be performed (19). Artiushin and colleagues recently observed a genomic diversity among different field isolates in an *M. hyopneumoniae*-specific PCR (1). Depending on the annealing temperature, the isolates could be divided into two separate groups. However, their target was an unknown part of the genome that might be prone to mutations. In this study we have targeted the 16S rRNA gene. As a part of the ribosome, with an overall central function, changes in rRNA are comparatively slow (15). This suggest that target mismatches can be excluded.

In a previous study *M. hyopneumoniae* was regularly isolated from pneumonic lesions and could also be recovered from pieces of the nasal mucosa. However, nasal washings proved to be less efficient than nasal mucosa for demonstrating the presence of *M. hyopneumoniae* (6). In those experiments no *M. hyopneumoniae* was recovered with nasal swabs. Our results in addition to these data suggest that very low levels of *M. hyopneumoniae* can be found in nose samples. This is also supported by the fact that it is extremely difficult to cultivate *M. hyopneumoniae* from nose samples (18). The presence of *M. hyopneumoniae* might also vary considerably during the course of infection. When the pigs were retested after 3 weeks, *M. hyopneumoniae* could be demonstrated only in one of five pigs previously PCR positive. However, it cannot be ruled out that what we observe actually is what is possible to recover from the upper part of the nose. In one of the pigs, *M. hyopneumoniae* was detected by PCR before the ELISA was positive. This suggests that it might be possible to detect *M. hyopneumoniae* prior to the humoral immunoresponse. However, a larger number of pigs have to be tested to confirm this observation.

Economical and animal health aspects are both important factors in the search of a way to control EP (18). The fastidious nature of *M. hyopneumoniae* calls for alternative methods for the detection of the organism. The advantage to using PCR is not only its rapidness but also the possibility to demonstrate presence of *M. hyopneumoniae* in live pigs prior to a humoral response to the microorganism. Interestingly, the PCR-positive pigs were clustered in one part of the herd (data not shown). Thus, through a combination of serological screening and PCR, valuable insights in the epidemiology of infections with *M. hyopneumoniae* might be achieved.

ACKNOWLEDGMENTS

We thank Göran Bölske for valuable discussions on porcine mycoplasmas.

This work was financially supported by a grant from the Swedish Farmers' Foundation for Agricultural Research.

REFERENCES

1. **Artiushin, S., L. Stipkovits, and F. C. Minion.** 1993. Development of polymerase chain reaction primers to detect *Mycoplasma hyopneumoniae*. Mol. Cell Probes **7:**381–385.
2. **Bölske, G.** 1988. Survey of mycoplasma infections in cell cultures and a comparison of detection methods. Zentralbl. Bakteriol. Hyg. A **269:**331–340.
3. **Bölske, G., M.-L. Strandberg, K. Bergström, and K.-E. Johansson.** 1987. Species-specific antigens of *Mycoplasma hyopneumoniae* and cross-reactions with other porcine mycoplasmas. Curr. Microbiol. **15:**233–239.
4. **Böttger, E. C.** 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. **65:**171–176.
5. **Friis, N. F.** 1975. Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*. Nord. Vet. Med. **27:**337–339.
6. **Goodwin, R. F. W.** 1972. Isolation of *Mycoplasma suipneumoniae* from the nasal cavities and lungs of pigs affected with enzootic pneumonia or exposed to this infection. Res. Vet. Sci. **13:**262–267.
7. **Goodwin, R. F. W., A. P. Pomeroy, and P. Whittlestone.** 1965. Production of enzootic pneumonia in pigs with a mycoplasma. Vet. Rec. **77:**1247–1249.
8. **Gray, M. W., D. Sankoff, and R. J. Cedergren.** 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Res. **12:**5837–5852.
9. **Harasawa, R., K. Koshimizu, O. Takeda, T. Uemori, K. Asada, and I. Kato.** 1991. Detection of *Mycoplasma hyopneumoniae* DNA by the polymerase chain reaction. Mol. Cell Probes **5:**103–109.
10. **Johansson, K.-E., J. G. Mattsson, K. Jacobsson, C. Fernández, K. Bergström, G. Bölske, P. Wallgren, and U. B. Göbel.** 1992. Specificity of oligonucleotide probes complementary to evolutionarily variable regions of 16S rRNA from *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. Res. Vet. Sci. **52:**195–204.
11. **Liesack, W., and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. **174:**5072–5078.
12. **Maniloff, J., R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.).** 1992. Mycoplasmas: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
13. **Mare, C. J., and W. P. Switzer.** 1965. New species: *Mycoplasma hyopneumoniae*, a causative agent of virus pig pneumonia. Vet. Med. **60:**841–846.
14. **Mattsson, J.** 1993. Stable RNA as a tool for DNA probes and phylogenetic studies. Ph.D. dissertation. Swedish University of Agricultural Sciences, Uppsala, Sweden.
15. **Olsen, G. J., and C. R. Woese.** 1993. Ribosomal RNA: a key to phylogeny. FASEB J. **7:**113–123.
16. **Rodwell, A. W., and R. F. Whitcomb.** 1983. Methods for direct and indirect measurement of mycoplasma growth. Methods Mycoplasmol. **1:**185–196.
17. **Ros Bascuñana, C., J. G. Mattsson, G. Bölske, and K.-E. Johansson.** 1994. Characterization of the 16S rRNA genes from *Mycoplasma* sp. strain F38 and development of an identification system based on PCR. J. Bacteriol **176:**2577–2586.
18. **Ross, R. F.** 1992. Mycoplasmal diseases, p. 537–551. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames.
19. **Saiki, R. K.** 1990. Amplification of genomic DNA, p. 13–20. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, San Diego, Calif.
20. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
21. **Stemke, G. W., F. Laigret, O. Grau, and J. M. Bové.** 1992. Phylogenetic relationships of three porcine mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and complete 16S rRNA sequence of *M. flocculare*. Int. J. Syst. Bacteriol. **42:**220–225.
22. **Stemke, G. W., R. Phan, T. F. Young, and R. F. Ross.** 1994. Differentiation of *Mycoplasma hyopneumoniae*, *M. flocculare* and *M. hyorhinis* on the basis of amplification of a 16S rRNA gene sequence. Am. J. Vet. Res. **55:**81–84.
23. **Svärd, S. G., J. G. Mattsson, K.-E. Johansson, and L. A. Kirsebom.** 1994. Cloning and characterization of the RNase P RNA genes from two porcine mycoplasmas. Mol. Microbiol. **11:**849–859.
24. **Taschke, C., K. Ruland, and R. Herrmann.** 1987. Nucleotide sequence data of the 16S rRNA of *Mycoplasma hyopneumoniae*. Nucleic Acids Res. **15:** 3918.
25. **Tully, J. G., J. M. Bové, F. Laigret, and R. F. Whitcomb.** 1993. Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic

cluster of arthropod-associated mollicutes to ordinal rank (*Entomoplasmatales* ord. nov.), with provision for familial rank to separate species with nonhelical morphology (*Entomoplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*. Int. J. Syst. Bacteriol. **43:**378–385.

26. **Wallgren, P., K. Artursson, C. Fossum, and G. V. Alm.** 1993. Incidence of infections in pigs bred for slaughter revealed by elevated serum levels of interferon and development of antibodies to *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae*. J. Vet. Med. B **40:**1–12.
27. **Wallgren, P., P. Beskow, C. Fellström, and L. H. M. Renström.** 1994. Porcine lung lesions at slaughter and their correlation to the incidence of infections with *Mycoplasma hypneumoniae* and *Actinobacillus pleuropneumoniae*. J. Vet. Med. B **41:**441–452.
28. **Wallgren, P., G. Bölske, and C. Fossum.** 1992. In vitro stimulation of antibody production to *Mycoplasma hyopneumoniae* by porcine peripheral blood mononuclear cells. Vet. Microbiol. **32:**363–374.
29. **Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts.** 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. **12:**219–286.
30. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
31. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171:**6455–6467.
32. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
33. **Woese, C. R., and G. E. Fox.** 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA **74:**5088–5090.