

## Detection of *Mycoplasma hyopneumoniae* in Bronchoalveolar Lavage Fluids of Pigs by PCR

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Received 30 October 1997/Returned for modification 4 February 1998/Accepted 9 April 1998

In the present investigation we developed a method for the detection of *Mycoplasma hyopneumoniae* in bronchoalveolar lavage fluid (BALF) of pigs by PCR with a primer pair flanking a DNA fragment of 853 bp specific for *M. hyopneumoniae*. Several methods were tested to eliminate the amplification inhibitors present in BALFs. The best results were obtained by the extraction of the DNA from the BALFs. By the PCR performed with the extracted DNA, 10<sup>2</sup> CFU of *M. hyopneumoniae* could be detected in 1 ml of BALF from specific-pathogen-free swine experimentally inoculated with *M. hyopneumoniae*. DNA from 11 other mycoplasma species and 17 cell-walled bacterial species colonizing the respiratory tracts of pigs was not amplified. In a field study BALFs from 40 pigs from farms with a history of chronic pneumonia were tested for *M. hyopneumoniae* by cultivation and by PCR (i) with BALFs incubated in Friis medium and (ii) with DNA extracted from the BALFs. In addition, PCR was performed with postmortem lung washings from 19 of the 40 pigs, and immunofluorescence tests were carried out with sections of lungs from 18 of the 40 pigs. *M. hyopneumoniae* could not be detected in 18 of the 40 pigs by any of the five methods tested. The remaining 22 pigs showed a positive reaction by the PCR with DNA extracted from the BALFs and variable positive reactions by the other tests. A complete correspondence could be observed between the immunofluorescence test result and the result of PCR with DNA. The investigation shows that the PCR with DNA extracted from BALFs is a suitable technique for the sensitive and specific in vivo detection of *M. hyopneumoniae*.

*Mycoplasma hyopneumoniae* is the primary agent of enzootic pneumonia of pigs (14, 22). The disease has a worldwide distribution and causes considerable economic losses in swine production due to reduced growth rate and feed conversion efficiency (24). The detection of *M. hyopneumoniae* is usually based on the isolation of the organisms by culture or by immunofluorescence tests with lung sections (3). The cultivation of *M. hyopneumoniae* is difficult due to the fastidious culture requirements and the extremely slow growth of *M. hyopneumoniae*, often resulting in overgrowth by other mycoplasmas colonizing the respiratory tracts of pigs (12). Cross-reactions with *Mycoplasma flocculare* and *Mycoplasma hyorhinis* reduce the specificity of conventional immunological detection methods (7).

With the development of PCR an alternative diagnostic method is now available. This method is suitable for the fast, sensitive, and specific detection of fastidiously growing microorganisms (5). Different PCR procedures have been described for the detection of *M. hyopneumoniae* (4, 6, 16, 30, 31), but none of these has been evaluated under field conditions. Only Mattsson et al. (23) and Verdin et al. (33) tested the PCRs that they developed with samples from fattening pigs, namely, with nasal swabs or tracheobronchiolar washings, respectively. The results obtained by PCR were compared with serological results but not with the results obtained by the classical antigen detection methods.

Previous studies demonstrated *M. hyopneumoniae* in lavage fluids of the respiratory system including bronchoalveolar lavage fluid (BALF) from living pigs (1, 25, 33). In the present investigation we developed a method for the detection of *M. hyopneumoniae* in BALF by PCR. Different sample preparations were tested for their ability to remove or inactivate amplification inhibitors present in BALFs. The PCR procedure was evaluated under field conditions, and the detection of *M. hyopneumoniae* by PCR was compared with that by culture and the immunofluorescence test.

### MATERIALS AND METHODS

**Organisms and growth conditions.** The mycoplasmas and walled bacteria used in this study are listed in Table 1. *Mycoplasma buccale*, *Acholeplasma axanthum*, and *Acholeplasma laidlawii* were cultivated in modified Hayflick-medium (18). *Mycoplasma suavis* was cultivated in the SP 4 medium described by Tully et al. (32), and the other mycoplasmas were cultivated in the medium developed by Friis (12) (Friis medium). The numbers of CFU of the mycoplasmas and the acholeplasmas were estimated by the method of Albers and Fletcher (2). Walled bacteria were grown in nutrient broth.

**DNA extraction.** The DNAs of the walled bacteria and of *M. hyopneumoniae* were obtained after incubation with lysis buffer (0.1 M NaCl, 10 mM Tris HCl [pH 8.0], 1 mM EDTA, 5% [vol/vol] Triton X-100, 2.8 mg of lysozyme per ml) and isopropanol precipitation as described by Sambrook et al. (27).

**BALF.** BALF was taken by fiberoptic bronchoscopy as described by Hensel et al. (17) and Ganter and Hensel (13). In short, the pigs were anesthetized with 2 mg of azaperone/kg of body weight<sup>-1</sup> intramuscularly and 15 mg of metomidate/kg<sup>-1</sup> intra-abdominally or with 10 mg of tiletamine-zolazepam (Tilest) kg<sup>-1</sup> intramuscularly and were positioned in a sling in sternal recumbency. After intubation the tip of the endoscope was placed in the bronchus trachealis supplying the right anterior lung lobe. Five portions of 20 ml of 0.15 M phosphate-buffered saline (PBS) were instilled and aspirated immediately.

**PCR.** An 853-bp fragment specific for the *M. hyopneumoniae* genome was amplified with the oligonucleotides H1 (5'-TAGAAATGACTGGCAGACAA-3') and H2 (5'-GAGGCTTGATTTGGAGTC-3'), which were used as primers. Amplification was carried out in a 50- $\mu$ l reaction mixture containing 10 mM Tris HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% (wt/vol) gelatin, 1.25% (vol/vol) formamide, each deoxynucleoside triphosphate at a concentration of

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TABLE 1. Bacterial strains used in the PCR specificity tests

Species	Strain	PCR results
<i>Mycoplasma hyopneumoniae</i>	J	+
<i>Mycoplasma hyopneumoniae</i>	241-4	+
<i>Mycoplasma hyopneumoniae</i>	257	+
<i>Mycoplasma hyopneumoniae</i>	724-2-3	+
<i>Mycoplasma arginini</i>	G230	-
<i>Mycoplasma buccale</i>	GH20274	-
<i>Mycoplasma flocculare</i>	Ms42	-
<i>Mycoplasma hyopharyngis</i>	H3-6BF	-
<i>Mycoplasma hyorhinis</i>	BTS7	-
<i>Mycoplasma hyosynoviae</i>	S16	-
<i>Mycoplasma sualvi</i>	Mayfield B	-
<i>Acholeplasma axanthum</i>	H86N	-
<i>Acholeplasma granularum</i>	BTS39	-
<i>Acholeplasma laidlawii</i>	PG8	-
<i>Acinetobacter calcoaticus</i>	Field strain	-
<i>Actinobacillus pleuropneumoniae</i>	Field strain	-
<i>Actinomyces pyogenes</i>	Field strain	-
<i>Bacillus subtilis</i>	Field strain	-
<i>Bordetella bronchiseptica</i>	Field strain	-
<i>Enterococcus faecalis</i>	Field strain	-
<i>Escherichia coli</i>	Field strain	-
<i>Haemophilus parasuis</i>	Field strain	-
<i>Klebsiella</i> spp.	Field strain	-
<i>Neisseria</i> spp.	Field strain	-
<i>Pasteurella multocida</i>	Field strain	-
<i>Proteus</i> spp.	Field strain	-
<i>Pseudomonas</i> spp.	Field strain	-
<i>Staphylococcus aureus</i>	Field strain	-
<i>Staphylococcus hyicus</i>	Field strain	-
<i>Streptococcus dysgalactiae</i> bv. <i>equisimilis</i>	Field strain	-
<i>Streptococcus suis</i> type 1	Field strain	-

<sup>a</sup> +, amplification; -, no amplification.

200 µM, 7.5 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Stratagene, Heidelberg, Germany). A hot start technique was used with Chill-out 14 liquid wax (Biozym, Hessisch Oldendorf, Germany) according to the manufacturer's instructions. After an initial denaturation step of 5 min at 94°C 50 cycles were performed in an automated thermal cycler (Perkin-Elmer Cetus, Langen, Germany). The samples were denatured at 93°C for 30 s. The primers were annealed at 55°C for 30 s and extended at 72°C for 90 s. The extension step of the last cycle was 10 min. Aliquots of the amplified sample (10 µl) were analyzed by electrophoresis in a 1% (wt/vol) agarose gel containing ethidium bromide (0.5 µg/ml).

To determine the specificity the PCR was performed with 10-µl cultures of the mycoplasmas and 1 µg of DNA of the walled bacteria listed in Table 1. The sensitivity of the PCR was determined by investigation of 10-fold dilutions of *M. hyopneumoniae* cultures or DNA.

**Preparation of BALFs for PCR.** Several methods were tested with BALFs from *M. hyopneumoniae*-free, specific-pathogen-free (SPF) swine inoculated with 10<sup>1</sup> to 10<sup>5</sup> CFU of *M. hyopneumoniae* per ml and mixed with 0.1 mg of dithiothreitol per ml. The mycoplasmas were harvested from 2-ml aliquots of the BALFs by centrifugation at 12,000 × g for 30 min. For the following preparations the pellets were suspended in 10 µl of distilled water.

(i) **Boiling and centrifugation.** The samples were inoculated into 100 µl of distilled water, boiled for 10 min, and centrifuged at 12,000 × g for 30 min. A total of 10 µl of the supernatant was used for PCR.

(ii) **Proteinase K digestion.** Samples were inoculated into 100 µl of proteinase K lysis buffer (100 µg of proteinase K [Pharmacia, Freiburg, Germany] per ml, 0.5% [vol/vol] Tween 20 [Merck, Darmstadt, Germany], 0.5% [vol/vol] Igpal [Sigma, Deisenhofen, Germany]) and were kept for 1 h at 60°C, according to the method of Campbell (8). The proteinase K was inactivated by boiling for 10 min. A total of 10 µl of the suspension was used for PCR.

(iii) **DNA extraction with the QIAamp blood kit.** The DNA was extracted according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany) and was precipitated with ethanol at 4°C overnight. DNA pellets were washed in 70% (vol/vol) ethanol and were resuspended in 20 µl of distilled water. A total of 1 µg of DNA was used for PCR.

(iv) **DNA extraction with the Nucleon II DNA extraction kit.** The DNA extraction with the Nucleon II DNA extraction kit (Scotlab, Wiesloch, Germany) was performed according to the manufacturer's instructions. In short, the samples were incubated in a solution containing 340 µl of reagent B (0.4 M Tris, 0.06 M EDTA, 0.15 M NaCl, 1% [wt/vol] sodium dodecyl sulfate) and 2.4 µl of RNase

A (10 mg/ml) for 30 min at 37°C. After incubation with 100 µl of 5 M sodium perchlorate for 15 min at room temperature and for 25 min at 65°C, the DNA was extracted with chloroform and precipitated with ethanol for 2 h at 4°C. The DNA pellets were washed with 70% (vol/vol) ethanol and were resuspended in 20 µl of distilled water. A total of 1 µg DNA was subjected to PCR.

(v) **DNA extraction by the CTAB method.** DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method described by Maass and Dalhoff (21). The samples were incubated in 600 µl of proteinase K lysis buffer (10 mM Tris-EDTA [pH 7.5] 0.5% [vol/vol] sodium dodecyl sulfate, 100 µg of proteinase K per ml) for 1 h at 60°C. After adding 100 µl of 5 M NaCl and 80 µl of CTAB (10% [wt/vol] in 0.7 M NaCl), the samples were incubated for 10 min at 65°C. The DNA was extracted with chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol at 4°C overnight. The DNA pellets were washed in 70% (vol/vol) ethanol and were resuspended in 20 µl distilled water. A total of 1 µg of DNA was used for PCR.

**Clinical specimens.** To evaluate the PCR procedure under field conditions, BALFs were taken from 40 pigs on 18 farms with problems of chronic pneumonia immediately before they were killed. All except one of the pigs showed macroscopic bronchopneumonic alterations in the lungs at necropsy. BALFs of pigs were investigated by cultivation and PCR. Lung samples from 19 randomly selected pigs were taken at necropsy and were investigated by the indirect immunofluorescence test for *M. hyopneumoniae* antigen. In addition, washings of the bronchus trachealis (bronchial washings) were obtained at necropsy from 18 of the 40 pigs. The tip of a catheter was placed in the bronchus trachealis supplying the right anterior lung lobe. Two portions of 1 ml of PBS (2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub> [pH 7.2]) were instilled into the bronchus and aspirated immediately. The two suspensions were combined and investigated by PCR.

**Cultivation of BALFs.** Immediately after collection, 1 ml of BALF was inoculated into 2 ml of Friis medium. For cultivation each 0.2 ml of this transport mixture were transferred to each of 1.8 ml of Friis medium and 1.8 ml of Friis medium containing 5% (vol/vol) rabbit anti-*M. hyorhinis* serum. Further dilutions (10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>) were produced by transferring 0.2 to 1.8 ml of medium. The tubes were incubated on a roller at 37°C up to 4 weeks and were investigated twice a week for a color change without turbidity. Cultures showing a color shift to yellow were subcultivated in liquid medium and on solid medium. The solid media were incubated at 37°C in a humid atmosphere with 5% CO<sub>2</sub> for at least 30 days and investigated twice a week for mycoplasma colonies. Differentiation was done by the immunobinding assay according to the method of Kotani and McGarrity (20) with rabbit antisera against *M. hyopneumoniae*, *M. hyorhinis*, *M. flocculare*, *Mycoplasma hyosynoviae*, and *A. laidlawii*.

**PCR with BALFs and postmortem lung washings.** PCR was performed with the BALFs incubated in Friis medium and with DNA extracted from the BALFs from the 40 pigs with a history of chronic pneumonia. To investigate the BALFs incubated in Friis medium by PCR, 10 µl was taken from the tubes prepared for cultivation (containing 1.8 ml of Friis medium and 0.2 ml of the transport mixture) after 3 days of incubation. For the PCR with the DNA of the BALFs, DNA was extracted from BALFs with the Nucleon kit and by the CTAB method. To ensure that the amplification inhibitors were eliminated by the preparation procedure, simulated positive samples from all BALFs collected in the field were prepared by adding 10<sup>4</sup> CFU of *M. hyopneumoniae*/ml of BALF and were investigated. In addition, PCR was performed with bronchial washings taken at necropsy from 18 of the 40 pigs. PCR was performed with DNA extracted from the washings by the CTAB method.

**Immunofluorescence test with lung sections.** Cryostat sections (3 to 4 µm) were obtained from two locations of pneumonic areas of the left apical lobe. In case *M. hyopneumoniae* was not detected at these two locations samples from two other locations were investigated. The sections were dried on the slides for 10 min at 37°C, fixed with acetone (20°C) for 10 min, incubated with rabbit anti-*M. hyopneumoniae* or rabbit anti-*Mycoplasma bovirhinis* serum (negative control), and diluted 1:300 with PBS containing 1% (vol/vol) swine serum for 30 min. After washing with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween) for 10 min, the sections were incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobuline (DAKO, Glostrup, Denmark) diluted 1:50 with PBS containing 1% (vol/vol) swine serum for 30 min. All incubation steps were performed at room temperature. The sections were then washed in PBS-Tween, covered with 90% (vol/vol) glycerol in 10% (wt/vol) barbital buffer, mounted with a cover glass, and investigated under UV light. Lung sections from pigs artificially infected with *M. hyopneumoniae* served as positive controls.

**Statistical analysis.** The sensitivities and specificities of the different methods of detection of *M. hyopneumoniae* were calculated in comparison with a "gold standard" (see Tables 3 and 4) as described by Fletcher et al. (11). The 95% confidence interval was estimated as described by Sachs (26). The Fisher exact test was performed with the software SAS (SAS, Heidelberg, Germany) to examine if the sensitivities or specificities of the detection methods differ significantly.

## RESULTS

**Specificity of the PCR.** A specific PCR amplification product of 853 bp was obtained from the type strain *M. hyopneumoniae*

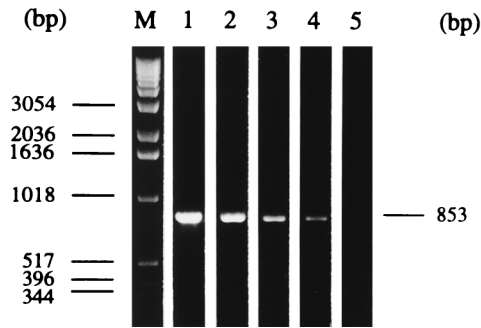


FIG. 1. Limit of detection of *M. hyopneumoniae* by PCR with DNA extracted from BALFs from SPF pigs inoculated with *M. hyopneumoniae*. BALFs from SPF pigs were inoculated with  $10^1$  to  $10^5$  CFU of *M. hyopneumoniae*/ml. DNA was extracted from 2-ml aliquots of these BALFs by the CTAB method. A total of 1  $\mu$ g of the DNA was used in the PCR. The amplification products were separated by agarose gel electrophoresis (1% agarose; 10  $\mu$ l sample per vial) and were visualized by ethidium bromide staining and with UV light. Lane M, molecular size marker (1-kb DNA ladder; Gibco BRL, Eggenstein, Germany); lane 1,  $10^5$  CFU of *M. hyopneumoniae*/ml of BALF from SPF pigs; lane 2,  $10^4$  CFU; lane 3,  $10^3$  CFU; lane 4,  $10^2$  CFU; lane 5, 10 CFU. The specific amplification product of 853 bp appears in lanes 1 to 4.

J and from the *M. hyopneumoniae* field strains. No amplification products appeared in the PCR with the other mycoplasmas and the walled bacteria investigated (Table 1). A minimum of  $10^3$  CFU of *M. hyopneumoniae* J/ml of culture or 10 fg of *M. hyopneumoniae* DNA could be detected.

**Sensitivity of the PCR.** Of the several BALF preparation methods tested, the best results were achieved by DNA extraction with the Nucleon II DNA extraction kit and by the CTAB method, allowing the detection of  $10^2$  CFU of *M. hyopneumoniae*/ml of BALF from SPF pigs (experimentally inoculated with *M. hyopneumoniae*) by PCR (Fig. 1). By the PCR with DNA extracted with the QIAamp blood kit, an amplification product was obtained only in the presence of at least  $10^3$  CFU of *M. hyopneumoniae*/ml of BALF. After boiling and centrifugation as well as after proteinase K digestion, the detection limit of the PCR was only  $10^5$  CFU of *M. hyopneumoniae*/ml of BALF.

**Detection of *M. hyopneumoniae* in BALFs of pigs with chronic pneumonia.** The Nucleon kit and the CTAB method were

used for the preparation of the BALFs obtained from the pigs with chronic pneumonia for PCR because of the highest sensitivity achieved by these two preparation methods in the PCR with BALFs from SPF pigs. In the treatment of the BALFs from pigs with pneumonia, these two preparation methods, however, had different effects on the PCR. These effects appeared in the PCR with the simulated positive samples. The removal of the amplification inhibitors succeeded by the CTAB method for 39 of the field-collected BALFs and with the Nucleon II DNA extraction kit for only 34 of the 40 field-collected BALFs.

The rates of detection of *M. hyopneumoniae* in pigs with chronic pneumonia obtained by cultivation, immunofluorescence tests, and PCR (i) with BALFs incubated in Friis medium, (ii) with DNA extracted from BALFs, and (iii) with washings from lungs are summarized in Table 2. *M. hyopneumoniae* could not be detected by any of the five methods tested in 18 of the 40 pigs investigated. All remaining 22 pigs showed a positive reaction in the PCR with DNA extracted from BALFs. The lungs of 11 of these 22 pigs were also examined by the immunofluorescence test. *M. hyopneumoniae* could be detected in all of them, indicating a complete correspondence of the results of these two methods. Of the 22 pigs showing *M. hyopneumoniae* by PCR with the DNA extracted from BALF, only 6 had a positive result by the PCR with BALFs incubated in Friis medium. The PCR with postmortem lung washings from 10 of these 22 pigs was positive for 6 pigs. The lowest detection rate was obtained by cultivation of the BALFs, which was positive for only 1 of the 40 pigs investigated. *M. hyorhinis* was isolated from 15 of the 40 pigs. The cultures of the BALFs from 13 pigs could not be evaluated because of the high level of contamination by walled bacteria. Mycoplasmas could not be cultivated from the BALFs of the remaining 11 pigs.

Table 3 presents the sensitivities and specificities, with confidence intervals, estimated for PCR with different samples, with PCR with DNA extracted from BALFs used as the apparent gold standard. PCR with cultures of BALFs and PCR with DNA extracted from bronchial washings showed by Fisher's exact test significantly lower sensitivities than that of the apparent gold standard.

The sensitivities and specificities of detection of *M. hyopneu-*

TABLE 2. Detection of *M. hyopneumoniae* in pigs with chronic pneumonia by cultivation, immunofluorescence test, and PCR

No. of pigs	Cultivation result	Immunofluorescence test result	Result of PCR with the following samples:		
			BALF incubated in Friis medium	DNA extracted from BALFs	DNA extracted from bronchial washings <sup>a</sup>
1	+ <sup>b</sup>	+	-	+	+
5	- <sup>c</sup>	+	-	+	+
4	-	+	-	+	-
1	-	+	+	+	NT
5	-	NT <sup>d</sup>	+	+	NT
6	-	NT	-	+	NT
10	-	NT	-	-	NT
7	-	-	-	-	-
1	-	-	-	? <sup>e</sup>	-
No. positive/no. investigated (%)	1/40 (2.5)	11/19 (58)	6/40 (15)	22/40 (55)	6/18 (30)

<sup>a</sup> Washings from lungs obtained at necropsy.

<sup>b</sup> +, positive reaction.

<sup>c</sup> -, negative reaction.

<sup>d</sup> NT, not tested.

<sup>e</sup> Not evaluable due to the presence of remaining amplification inhibitors.

TABLE 3. Sensitivities and specificities of detection of *M. hyopneumoniae* by PCR with different samples in comparison to those of detection by PCR with DNA extracted from BALFs<sup>a</sup>

Sample used for PCR	Sensitivity (% [95% confidence interval])	Specificity (% [95% confidence interval])
DNA extracted from BALFs (CTAB method) ( <i>n</i> = 40) <sup>b</sup>	100	100 B
Cultures of BALFs ( <i>n</i> = 40)	27 A (11–50)	100 B (81–100)
DNA extracted from postmortem bronchial washings (CTAB method) ( <i>n</i> = 18)	60 A (26–88)	100 B (63–100)

<sup>a</sup> Groups sharing letters are not significantly different by Fisher's exact test ( $P < 0.05$ ).

<sup>b</sup> This sample and its results represent the gold standard.

*moniae* by culture and PCR in comparison to those of detection by the immunofluorescence test are presented in Table 4.

## DISCUSSION

In the present investigation a PCR procedure and a sample preparation method for the detection of *M. hyopneumoniae* in BALFs have been developed, and the results were compared with those of the detection of *M. hyopneumoniae* by culture and an immunofluorescence test. The PCR procedure described here proved to be specific and sensitive for the detection of *M. hyopneumoniae*.

BALFs contain large amounts of substances inhibiting the PCR, and these substances must be removed before PCR. Several methods were tested for their suitability at removing the amplification inhibitors from BALFs from SPF pigs inoculated with different amounts of *M. hyopneumoniae*. In these investigations boiling and centrifugation as well as digestion with proteinase K appeared to be unsuitable methods since the sensitivity of detection of *M. hyopneumoniae* in the subsequent PCR was very low. This is in agreement with the results of other studies, which showed that lysis of BALFs by boiling and detergent treatment and subsequent centrifugation cannot remove all inhibitory substances (9) and that polysaccharides and lipids which are not degraded by proteinase K can still act as amplification inhibitors (15, 19, 21, 28).

In the investigation of BALFs from SPF swine, the highest sensitivity was observed with the PCR with DNA extracted with the Nucleon kit and by the CTAB method. Less sensitive was the PCR with DNA from BALFs extracted with the QIAamp blood kit, probably due to insufficient removal of the

amplification inhibitors. The limit of detection of *M. hyopneumoniae* by PCR in field samples may be somewhat lower than the sensitivity determined with BALFs from SPF pigs due to a larger amount of foreign DNA originating from purulent inflammations and the possible presence of additional inhibitory substances in BALFs from clinically affected swine (14, 28). Although identical regarding the sensitivity of the PCR with BALFs from SPF swine, the Nucleon kit and the CTAB method differed in their abilities to remove amplification inhibitors from the BALFs from pigs with respiratory problems. The present investigations showed a clear superiority of the CTAB method regarding the removal of amplification inhibitors from the BALFs from the clinically affected swine.

In the field study the highest rate of detection of *M. hyopneumoniae* was achieved by the PCR with DNA extracted from the BALFs from the pigs. PCR with DNA extracted from bronchial washings from the lungs at autopsy yielded fewer positive results. This may be the result of contamination with blood, which could act as an additional inhibitor (10, 29) and which could increase the concentration of nonmycoplasma DNA. The lowest sensitivity was obtained by the PCR with BALFs cultivated in Friis medium. Apparently, the field strains of *M. hyopneumoniae* did not multiply in these cultures and the amplification inhibitors in the medium (for example, the serum content) as well as the fact that the target DNA was not enriched by centrifugation (as was done with BALFs investigated by PCR with extracted DNA) prevented amplification of the small amount of the DNA of the organisms present. *M. hyopneumoniae* did not multiply under the cultivation conditions used, and this also occurred in the cultivation of the BALFs, in which *M. hyopneumoniae* could be detected in only 1 of the 22 BALFs that were *M. hyopneumoniae* positive by the PCR. Because of its fastidious cultivation requirements *M. hyopneumoniae* is generally difficult to cultivate, and it appears that it is particularly hard to cultivate it from BALFs. This was also reported by Mattsson et al. (23), who could cultivate *M. hyopneumoniae* from lungs but not from BALFs.

A complete correlation between the results obtained by PCR and the results obtained by the immunofluorescence test with lung sections was observed in the present investigations, indicating the similar sensitivities of both of these methods. While the immunofluorescence test, however, is restricted to the investigation of dead animals, the PCR with BALF can also be performed with samples from living pigs.

In conclusion, the PCR with DNA extracted from BALFs by the CTAB method by the procedure described here appears to be a suitable method for the detection of *M. hyopneumoniae* in living pigs. Due to its high sensitivity, even small amounts of organisms, characteristic of early stages of infection and sub-clinical infection, can be detected by this procedure, and importantly, noncultivable strains of *M. hyopneumoniae* can also be detected by this method. BALFs can be collected from living pigs, allowing random sampling of a representative number of diseased as well as healthy animals of a herd. With the PCR with the BALFs, reliable and fast information about the occurrence of *M. hyopneumoniae* in a pig herd can be obtained.

## ACKNOWLEDGMENTS

We thank P. Ahrens for providing the oligonucleotide sequences and multiple valuable suggestions. We thank also N. F. Friis for providing the field strains of *M. hyopneumoniae* and helpful advice concerning the cultivation of *M. hyopneumoniae*.

## REFERENCES

1. Abiven, P., and P. Pommier. 1993. Technique de lavage tracheobronchique par la voie transnasal pour la détection de *Mycoplasma hyopneumoniae* chez

TABLE 4. Sensitivities and specificities of detection of *M. hyopneumoniae* by culture and PCR in comparison to those of detection by the immunofluorescence test<sup>a</sup>

Examination method	Sensitivity (% [95% confidence interval])	Specificity (% [95% confidence interval])
Immunofluorescence test ( <i>n</i> = 19) <sup>b</sup>	100 A	100 B
Culture ( <i>n</i> = 19)	9 (0.2–41)	100 B (63–100)
PCR with DNA extracted from BALFs ( <i>n</i> = 19)	100 A (72–100)	100 B (63–100)

<sup>a</sup> Groups sharing letters are not significantly different by Fisher's exact test ( $P < 0.05$ ).

<sup>b</sup> This test and its results represent the gold standard.

- le porc vivant non anesthésié. *Vet. Res.* **24**:515–522.
2. Albers, A. C., and R. D. Fletcher. 1982. Simple method for quantitation of viable mycoplasmas. *Appl. Environ. Microbiol.* **43**:958–960.
  3. Armstrong, C. H., A. B. Scheidt, H. L. Thacker, L. J. Runnels, and M. J. Freeman. 1984. Evaluation of criteria of the postmortem diagnosis of mycoplasmal pneumonia of swine. *Can. J. Comp. Med.* **48**:278–281.
  4. 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.
  5. Bej, A. K., M. H. Mahubani, and R. M. Atlas. 1991. Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications. *Crit. Rev. Biochem. Mol. Biol.* **26**:301–334.
  6. Blanchard, B., P. Abiven, C. Saillard, M. Kobisch, and J. M. Bové. 1992. Specific detection of *Mycoplasma hyopneumoniae* in clinical samples, p. 12. In *Proceedings of the 6th International Symposium of ADILA and O.I.E.*
  7. Bölske, G., M.-L. Strandberg, K. Bergström, and K.-E. Johansson. 1987. Species-specific antigen of *Mycoplasma hyopneumoniae* and cross-reaction with other porcine mycoplasmas. *Curr. Microbiol.* **15**:233–239.
  8. Campbell, L. A. 1993. PCR detection of *Chlamydia pneumoniae*, p. 247–252. In D. H. Persing, T. S. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic molecular microbiology: principles and applications*. American Society for Microbiology, Washington, D.C.
  9. Clarridge, J. E., R. M. Shawar, T. M. Shinnick, and B. B. Plikaytis. 1993. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J. Clin. Microbiol.* **31**:2049–2056.
  10. De Francis, R., N. C. P. Cross, N. S. Foulkes, and T. M. Cox. 1988. A potent inhibitor of *Taq* polymerase copurifies with human genomic DNA. *Nucleic Acids Res.* **16**:10355.
  11. Fletcher, R. H., S. W. Fletcher, and E. W. Wagner. 1982. Diagnosis, p. 42–75. In *Clinical epidemiology. The essentials*. The Williams & Wilkins Co., Baltimore, Md.
  12. Friis, N. F. 1975. Some recommendations concerning primary isolation of *Mycoplasma suis pneumoniae* and *Mycoplasma flocculare*. *Nord. Veterinaermed.* **27**:337–339.
  13. Ganter, M., and A. Hensel. 1997. Cellular variables in bronchoalveolar lavage fluids (BALF) in selected healthy pigs. *Res. Vet. Sci.* **63**:215–217.
  14. 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.
  15. Greenfield, L., and T. J. White. 1993. Sample preparation methods, p. 122–137. In D. H. Persing, T. S. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic molecular microbiology: principles and applications*. American Society for Microbiology, Washington, D.C.
  16. 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.
  17. Hensel, A., M. Ganter, S. Kipper, S. Krehon, M. M. Wittenbrink, and K. Petzoldt. 1994. Prevalence of aerobic bacteria in bronchoalveolar lavage fluid from healthy pigs. *Am. J. Vet. Res.* **55**:1697–1702.
  18. Kirchoff, H., and R. Rosengarten. 1984. Isolation of a motile mycoplasma from fish. *J. Gen. Microbiol.* **130**:2439–2445.
  19. Kolk, A. H. J., A. R. J. Schuitema, S. Kuijper, J. van Leeuwen, P. W. M. Hermans, J. D. A. van Embden, and R. A. Hartskeerl. 1992. Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. *J. Clin. Microbiol.* **30**:2567–2575.
  20. Kotani, H., and G. J. McGarrity. 1985. Identification of mycoplasma colonies by immunobinding. *J. Clin. Microbiol.* **23**:783–785.
  21. Maass, M., and K. Dalhoff. 1994. Comparison of sample preparation methods for the detection of *Chlamydia pneumoniae* in bronchoalveolar lavage fluid by PCR. *J. Clin. Microbiol.* **32**:2616–2619.
  22. Maré, C. J., and W. P. Switzer. 1965. New species: *Mycoplasma hyopneumoniae*—a causative agent of virus pig pneumonia. *Vet. Med.* **60**:841–846.
  23. Mattsson, J. G., K. Bergström, P. Wallgren, and K.-E. Johansson. 1995. Detection of *Mycoplasma hyopneumoniae* in nose swabs from pigs by in vitro amplification of the 16S rRNA gene. *J. Clin. Microbiol.* **33**:893–897.
  24. Ross, R. F. 1992. Mycoplasmal diseases, p. 537–551. In A. D. Leman, R. D. Click, W. L. Mengerling, R. H. C. Penny, E. Scholl, and B. Straw (ed.), *Diseases of swine*. Iowa State University Press, Ames.
  25. Runge, M., M. Ganter, F. Delbeck, W. Hartwick, A. Ruffer, B. Franz, and U. G. Amtsberg. 1996. Etiological diagnosis of pneumonia in fattening pigs: investigations of bronchoalveolar lavage (BAL) by culture methods and immunofluorescence microscopy as well as serological findings. *Berl. Münch. Tierärztl. Wochenschr.* **109**:101–107.
  26. Sachs, L. 1992. Die Häufigkeit von Ereignissen, p. 432–443. In *Angewandte Statistik*. Springer Verlag, Berlin, Germany.
  27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  28. Skakni, L., A. Sardet, J. Just, J. Landman-Parker, J. Costil, N. Moniot-Ville, F. Bricout, and A. Garbarg-Chenon. 1992. Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. *J. Clin. Microbiol.* **30**:2638–2643.
  29. Soini, H., M. Skurnik, K. Lippo, E. Tala, and M. K. Viljanen. 1992. Detection and identification of mycobacteria by amplification of a segment of the gene coding the 32-kilodalton protein. *J. Clin. Microbiol.* **30**:2025–2028.
  30. Sørensen, V., P. Ahrens, K. Barford, A. A. Feenstra, N. C. Feld, N. F. Friis, V. Bille-Hansen, N. E. Jensen, and M. W. Pedersen. 1997. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four different assays. *Vet. Microbiol.* **54**:23–34.
  31. 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.
  32. Tully, J. G., R. F. Whitcomb, H. F. Clark, and D. L. Williamson. 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* **195**:892–894.
  33. Verdin, E., B. Blanchard, M. Kobisch, J. M. Bové, and C. Saillard. 1996. Use of nested PCR diagnosis test to detect *Mycoplasma hyopneumoniae* under field conditions. *IOM Lett.* **4**:101–102.