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

A. KATRIN BAUMEISTER, MARTIN RUNGE, MARTIN GANTER, ANNE A. FEENSTRA, FRIEDRICH DELBECK, AND HELGA KIRCHHOFF

Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Hannover, and Außenstelle für Epidemiologie, Tierärztliche Hochschule Hannover, Bakum, Germany, and Danish Veterinary Laboratory, Copenhagen, Denmark

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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, 10^2 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 amplified with the oligonucleotides H1 (5'-GAGGCTTTGATTTTGGAGTC-3') 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 amplified with the oligonucleotides H1 (5'-GAGGCTTTGATTTTGGAGTC-3'), which were used as primers. Amplification was carried out in a 50-μl reaction mixture containing 10 mM Tris HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl_2, 0.01% (wt/vol) gelatin, 1.25% Tris buffered saline (PBS) were instilled and aspirated immediately.

**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 sualvi* 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).

**PCR.** 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 i.v. intramuscularly and 15 mg of metamizode kg i.v., intra-abdominally or with 10 mg of tiletamine-zolazepam (Tilest) kg i.v. 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'-TAGAAATGACTGGCAGACA-3') and H2 (5'-GACGGCTTTGATTTTGGAGTC-3'), which were used as primers. Amplification was carried out in a 50-μl reaction mixture containing 10 mM Tris HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl_2, 0.01% (wt/vol) gelatin, 1.25% (vol/vol) formamide, each deoxynucleoside triphosphate at a concentration of...
A (10 mg/ml) for 30 min at 37°C. After incubation with 100 μl of 5 M sodium phosphate 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 Maas and Dalhoff (21). The samples were incubated in 600 μl of proteinase K lysis buffer (10 mM Tris-HCl, 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-isooamyl alcohol (24:1) and phenol-chloroform-isooamyl 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 of 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 KH2PO4, 137 mM NaCl, 8 mM Na2HPO4, [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 was 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 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 KH2PO4, 137 mM NaCl, 8 mM Na2HPO4, [pH 7.2]) were instilled into the bronchus and aspirated immediately. The two suspensions were combined and investigated by PCR.

PCR with BALFs and postmortem lung washings. PCR was performed with the BALFs incubated in Friis medium and with DNA extracted from the BALFs. Cultivation was performed on 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 105 CFU of M. hyopneumoniae/ml of BALF and were investigated. In addition, PCR was performed with bronchial washings taken at the same time.

**Immunofluorescence test with lung sections.** Cryostat sections (3 to 4 μm) were cut from two locations of pneumonic areas of the left apical lobe. In one 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 bovis serum (negative control), and diluted 1:300 with PBS containing 1% (vol/vol) 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 immunoglobulin G (DAKO, Glostrup, Denmark) diluted 1:50 with PBS containing 1% (vol/vol) 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% (vol/vol) barbital buffer, mounted with a cover glass, and investigated under UV light. Lung sections from pigs artifically 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 10^7 to 10^9 CFU of M. hyopneumoniae/ml. DNA was extracted from 2-ml aliquots of these BALFs by the CTAB method. A total of 1 μg of the DNA was used in the PCR. The amplification products were separated by agarose gel electrophoresis (1% agarose; 10 μl 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/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 10^2 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>
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<tr>
<th>No. of pigs</th>
<th>Cultivation result</th>
<th>Immunofluorescence test result</th>
<th>Result of PCR with the following samples:</th>
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<tr>
<td>BALF incubated in Friis medium</td>
<td>DNA extracted from BALFs</td>
<td>DNA extracted from bronchial washings</td>
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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.
DNA extracted from postmortem cultures of BALFs (n = 5) was used for PCR with DNA extracted from BALFs with the Nucleon kit and with the CTAB method. Less sensitive lipids which are not degraded by proteinase K can still act as 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 nonmycoplasmal 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 subclinical 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.

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**REFERENCES**

1. Ahiven, P., and P. Pommier. 1993. Technique de lavage trachéobronchique par la voie transnasal pour la détection de Mycoplasma hyopneumoniae chez