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, 10^2 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 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 PCRds that they developed with samples from fattening pigs, namely, with nasal swabs or tracheobronchial 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 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).

BALF. BALF was taken by fiberoptic bronchoscopy as described by Hensel et al. (17) and Gaertner and Hensel (13). In short, the pigs were anesthetized with 2 mg of azaperone kg of body weight 1 intramuscularly and 15 mg of metomidate kg 2 intravenously. The tracheas were cannulated and were positioned in a sling in sternal recumbency. After Intubation the tip of the endoscope was placed in the bronchus trachealis superior and inferior, and 9 ml of warmed 0.9% NaCl solution were instilled and aspirated immediately.

PCR. An 853-bp fragment specific for the *M. hyopneumoniae* genome was amplified with the oligonucleotides H1 (5'-TAGAATAGTACTGACAGCAACA-3') and H2 (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 MgCl₂, 0.01% (wt/vol) gelatin, 1.25% (vol/vol) formamide, each deoxynucleoside triphosphate at a concentration of 10 μM, and 1.25 U of Taq polymerase (Pharmacia, Sweden).

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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 cetlytrimethylammonium 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-isooamyl alcohol (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 changes in the lungs at necropsy. BALFs of the pigs were investigated by cultivation and PCR. Last 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 bronchi trachealis (bronchial washings) were obtained at necrosis from 18 of the 40 pigs. The tip of a catheter was placed in the bronchus trachealis supplying the right anterior 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 bronchi 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 Fries medium. For cultivation each 0.2 ml of this transport mixture was transferred to each of 1.8 ml of Fries medium and 1.8 ml of Fries medium, containing 10% (vol/vol) rabbit anti-*M. hyorhinis* serum. Further dilutions (10−2, 10−3, 10−4) 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% CO2 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 McGavin (20) with rabbit antiserum against *M. hyorhinis*, *M. hyopneumoniae*, *M. flocculare*, *Mycoplasma hyorhinis*, and *A. laidlawii*.

**PCR with BALFs and postmortem lung washings.** PCR was performed with DNA from the BALFs incubated in Fries medium and with DNA extracted from the BALFs of the 40 pigs with a history of chronic pneumonia. To investigate the BALFs incubated in Fries medium by PCR, 10 µl was taken from the tubes prepared for cultivation (containing 1.8 ml of Fries 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 104 CFU of *M. hyopneumoniae/ml of BALF* and were investigated in addition. PCR was performed with bronchial washings taken at the lungs 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 cut from two locations of pneumonia affected lung tissue. 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) 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 antirabbit immunoglobulin (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% (vol/vol) barbital barbital, 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*.
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 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.

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

<table>
<thead>
<tr>
<th>No. of pigs</th>
<th>Cultivation result</th>
<th>Immunofluorescence test result</th>
<th>Result of PCR with the following samples:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BALF incubated in Friis medium</td>
</tr>
<tr>
<td>1</td>
<td>+(^b)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>–(^c)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>NT(^d)</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>NT(^d)</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>NT(^d)</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

No. positive/no. investigated (%) 1/40 (2.5) 11/19 (58) 6/40 (15) 22/40 (55) 6/18 (30)

\(^a\) Washings from lungs obtained at necropsy.

\(^b\) +, positive reaction.

\(^c\) –, negative reaction.

\(^d\) NT, not tested.

\(^e\) 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

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

*a* Groups sharing letters are not significantly different by Fisher’s exact test \((P < 0.05)\).

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 unsufficient 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 inflamations 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.

**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*.

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