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

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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 tested. By the PCR performed with BALFs from 40 pigs from farms with a history of chronic pneumonia were 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 fast, sensitive, and specific for the fastidious, fast-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 PCR against 11 other mycoplasma species and 17 cell-walled bacterial species colonizing the respiratory tracts of pigs (12). Cross-reactions with other mycoplasmas and walled bacteria in the respiratory tract of pigs (12). Cross-reactions with *Mycoplasma flocculare* and *Mycoplasma hyorhinis* reduce the specificity of conventional immunological detection methods (7).

PREVIOUS STUDIES DETERMINED THAT AN 853-BP FRAGMENT SPECIFIC FOR THE *M. hyopneumoniae* genome was amplified with the oligonucleotides Hi (5'-TAGAATTGTACTGCGACAAAA-3') and H2 (5'-GGGCCTTACGGTGGAGTC-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, 0.1% (wt/vol) gelatin, 1.25% (vol/vol) formamide, each deoxynucleoside triphosphate at a concentration of 10 mM, 1.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 0.2 μM of each primer. 100 μl of the PCR product was mixed with 900 μl of DNA extraction. The 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. 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*.

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. (52), 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 DNA from the walled bacteria and of *M. hyopneumoniae* was 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 -1 intramuscularly and 15 mg of metamitridal kg -1 intra-abdominally or with 10 mg of tiletamine-zolazepam (Telzil) kg -1 intramuscularly and were positioned in a sling in sternal recumbency. After intubation the tip of the endoscope was placed in the bronchus trachealis sup-
M EDTA, 0.15 M NaCl, 1% (wt/vol) sodium dodecyl sulfate) and 2.4 M 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 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 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 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 the bronchi trachealis 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 bronchi and aspirated immediately. The two suspensions were combined and inoculated into the field condition. The BALFs were immediately collected, 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 (10-2, 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 McNeilly (20) with rabbit antisera against M. hyorhinis, M. hyopneumoniae, M. flocculare, Mycoplasma hyorhinis, 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 of 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 106 CFU of M. hyopneumoniae/ml of BALF and were investigated. In addition, PCR was performed with bronchial washings taken at autopsy 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 μm) were cut from two locations of pneumatic areas of the lung. 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-Mycoplasm bovides serum (negative control), and diluted 1:30 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 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% (wt/vol) barbitral 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 the 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.
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>
<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>
</table>
|             |                   |                                | BALF incubated in Friis medium | DNA extracted from BALFs | DNA extracted from bronchial washings

1/40 (2.5) 11/19 (58) 6/40 (15) 22/40 (55) 6/18 (30)

Not evaluable due to the presence of remaining amplification inhibitors.
Cultures of BALFs (PCR with DNA extracted from BALFs) showed a sensitivity of 100% compared to 100% specificity. This is in agreement with the results of other studies, which showed that lysis of BALFs by boiling and PCR was very low. This is in agreement with the results of investigations boiling and centrifugation as well as digestion with proteinase K appeared to be unsuitable methods since the sensitivity of detection of M. hyopneumoniae BALFs have been developed, and the results were compared to those of detection by culture and PCR in comparison to those of detection by the immunofluorescence test are presented in Table 4.

### 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>

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

**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 still can 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 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 nonmucoplasma 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.

### TABLE 4. Sensitivities and specificities of detection of M. hyopneumoniae by culture and PCR in comparison to those of detection by the immunofluorescence test

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

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

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