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A Multiplex PCR to Identify Porcine Mycoplasmas Present in Broth Cultures

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ABSTRACT

Mycoplasma hyopneumoniae, *Mycoplasma hyorhinis* and *Mycoplasma flocculare* can be present in the lungs of pigs at the same time. These three mycoplasma species all require similar growth conditions and can be recovered from clinical samples using the same media. We have developed a multiplex PCR as a helpful tool for rapid differentiation of these three species in the course of isolation. Based on the 16S ribosomal DNA sequences, three different forward primers and a single reverse primer were selected. Each forward primer was compared to available mycoplasma sequences, showing the primers to be specific. The three amplification products observed of 1129 bp (*M. hyorhinis*), 1000 bp (*M. hyopneumoniae*) and 754 bp (*M. flocculare*) were clearly distinguishable on a 1% agarose gel. In addition, no cross-reaction with *Mycoplasma hyosynoviae*, another porcine mycoplasma, was noted. This multiplex PCR using the proposed set of primers is the first reported assay that allows the simultaneous identification of the different *Mycoplasma* species isolated from the lungs of pigs.

Keywords: 16S ribosome DNA, isolation, multiplex, mycoplasma, *M. flocculare, M. hyopneumoniae*, *M. hyorhinis*, PCR, swine

Abbreviations: BLAST, Basic Local Alignment Search Tool; CCU, colour-changing units; DFVF, Danish Institute for Food and Veterinary Research

INTRODUCTION

Mycoplasma hyopneumoniae is the primary pathogen involved in enzootic pneumonia and is among the most prevalent agents associated with the porcine respiratory disease complex. Despite the enormous economic impact of the disease (Maes *et al.*, 1999), fundamental research is limited owing to demanding isolation techniques. Epidemiological studies are hampered by difficulties in detecting *M. hyopneumoniae* strains in pig herds. Recently, several nested PCR assays have been developed for direct detection on clinical or environmental samples, but they are unable to discriminate between viable and non-viable microorganisms (Stärk *et al.*, 1998; Calsamiglia *et al.*, 1999a). The particular benefit of this technique is its high sensitivity, but extra care is needed since the risk of contamination is much higher compared to standard PCR

methods. In addition, a number of reports indicate the presence of inhibitory components that may yield false-negative results when working on clinical samples instead of purified DNA (Wiedbrauk *et al.*, 1995; Lantz *et al.*, 2000). Enzyme linked immunosorbent assays for the detection of antibodies have also been used (Feld *et al.*, 1992; Sørensen *et al.*, 1996). Owing to delay and differences in time of seroconversion as well as interference of maternal antibodies in young piglets (Morris *et al.*, 1994), serological results must be interpreted with care. *Mycoplasma hyorhinis*, on the other hand, may cause serofibrinous to fibrinopurulent polyserositis and arthritis, but is also frequently isolated from the respiratory tract of healthy pigs. *M. flocculare* has not been linked to any disease so far, but the organism is widely spread in swine as well (Kobisch and Friis, 1996). For these economically less important porcine *Mycoplasma* spp., even fewer diagnostic kits are available. Therefore, isolation, albeit labour-intensive and limited in sensitivity, remains the gold standard for the diagnosis of porcine *Mycoplasma* infections (Friis, 1971a,b, 1975, 1979; Goodwin, 1972; Kobisch and Friis, 1996).

M. hyopneumoniae, *M. hyorhinis* and *M. flocculare* have been reported to cross-react serologically (Bölske *et al.*, 1987; Strasser *et al.*, 1992) and exhibit extended phylogenetic similarities (Stemke *et al.*, 1992). The three species are able to grow in the same media and can complicate unambiguous diagnosis. A fourth porcine mycoplasma, namely *M. hyosynoviae*, is associated with arthritis in domestic pigs, but it has other nutritive requirements. In contrast to the other three *Mycoplasma* species, this bacterium is grown in media enriched with arginine (Friis, 1974). Different techniques, including PCR, have already been reported to differentiate these porcine *Mycoplasma* species (Dussurget and Roulland-Dussoix, 1994; Stemke *et al.*, 1994; Mattsson *et al.*, 1995; Blanchard *et al.*, 1996; Baumeister *et al.*, 1998; Caron *et al.*, 2000; Boye *et al.*, 2001), but no single PCR test has been described that simultaneously distinguishes *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare*. The aim of this study was to develop such a multiplex PCR to identify these *Mycoplasma* species in broth culture. The use of a multiplex PCR for a rapid differentiation between these species would save time and money.

MATERIALS AND METHODS

Mycoplasma strains and cultivation

The reference strains used in this study were the *M. hyopneumoniae* J strain (ATCC 25934), the *M. flocculare* Ms42 strain (ATCC 27399) and the *M. hyorhinis* BTS-7 strain (ATCC 17981), all kindly provided by Professor N. Friis (Danish Institute for Food and Veterinary Research (DFVF), Copenhagen, Denmark). Purified DNA of the *M. hyosynoviae* S16 reference strain (ATCC 25591) was kindly provided by Dr B. Kokotovic (DFVF, Copenhagen, Denmark). Five *M. hyopneumoniae*, five *M. hyorhinis* and five *M. flocculare* field strains, all isolated from the lungs of Belgian pigs, were also included in this study. Cultivation of these mycoplasmas was performed similarly to that in earlier reports (Friis, 1971b, 1975, 1979). Briefly, basal broth

medium was composed of 2500 ml Hanks balanced salt solution, 1400 ml MilliQ H_2O, 15 g brain heart infusion (Difco, Detroit, MI, USA) and 16 g PPLO broth (Difco). The mixture was autoclaved at 121°C for 2 min and 180 ml of YCS-2 yeast extract (Sigma, St. Louis, MO, USA), 800 mg bacitracin (Sigma), 500 mg ampicillin (Sigma) and 10 ml of a sterile 0.6% phenol red solution were added. Horse serum and pig serum were filter-sterilized and added to the basal broth medium before use. The final medium contained 80% basal medium, 10% horse serum and 10% pig serum. The pH was adjusted to 7.35 using HCl. Growing cultures showed a gradually progressive colour change from red to yellow.

Sample preparation method

Genomic DNA of the reference strains was prepared using a phenol–chloroform extraction as described earlier (Miles and Nicholas, 1998). Since this method is labour intensive, other methods were tested on five field isolates of each of the three species. The isolates were grown before processing to a point were the broth had changed to an orange to yellow colour. In a first approach, 1 µl of the medium with mycoplasmas was used directly in the multiplex PCR. In a second approach, 1 ml of the growing cultures was spun down (2 min, 10 000*g*) and the pellets were resuspended in 100 µl sterile water. After boiling for 5 min, the samples were cooled on ice and spun down again. One µl of the supernatant was used as a template. In a third method, the mycoplasmas were spun down and resuspended as in the second method, but were then incubated in the presence of 2 U of proteinase K for 2 h at 37°C. Next, the proteinase K was inactivated at 65°C for 20 min and 1 µl of the mixture was used as a template during PCR. In a final sample preparation method, 1 ml of the broth cultures was spun down and resuspended in 50 µl lysis buffer (0.25% SDS in 0.05 mol/L NaOH). After 5 min at 95°C, the mixture was cooled and diluted with 250 µl sterile water. Again 1 µl of the supernatant was used as a template during PCR.

Selection of primers and multiplex reaction

Three specific forward primers were selected based on the aligned 16S rDNA sequences of *M. hyopneumoniae* (GenBank accession no. EO2783), *M. flocculare* (GenBank accession no. X63377), and *M. hyorhinis* (GenBank accession no. M24658). One common reverse primer was selected in a conserved region of the aligned 16S rRNA genes. Based on these sequences, the theoretical amplification products are 1000 bp (*M. hyopneumoniae*), 754 bp (*M. flocculare*), and 1129 bp (*M. hyorhinis*) in length. The different primers (listed in Table I) were combined in a single multiplex reaction. Thirty cycles (30 s at 94°C; 15 s at 54.6°C; and 1 min at 68°C) were run on a GeneAmp 9600 Thermal Cycler (Perkin Elmer, Wellesley, MA, USA) using 2.5 U recombinant *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands), 1 × Taq buffer, 75 nmol $MgCl_2$, 10 nmol of each dNTP, 8 pmol of each forward and 12 pmol of the reverse primer. The multiplex reaction was tested on purified DNA of

TABLE I
Primers used in the multiplex PCR

Primer name	Sequence	GC%	Length
M HYOP FOR	5′ TTCAAAGGAGCCTTCAAGCTTC 3′	45.5	22
M FLOC FOR	5′ GGGAAGAAAAAAATTAGGTAGGG 3′	39.1	23
M HYOR FOR	5′ CGGGATGTAGCAATACATTCAG 3′	45.5	22
M REV	5′ AGAGGCATGATGATTTGACGTC 3′	45.5	22

the reference strains. To examine the simultaneous detection of the *Mycoplasma* species, a DNA sample mix containing 2 ng genomic DNA of each species was included as well. Besides working on purified genomic DNA, the multiplex PCR was validated starting directly from growing cultures using the DNA template preparation methods described above.

Specificity

The specificity of the primers was determined using the Basic Local Alignment Search Tool (BLAST V2.8.9 (2 191 424 sequences); Altschul *et al.*, 1990). BLAST searches showed the primers to be highly specific among mycoplasmas. No other mycoplasmal sequence, except for those under investigation, completely matched the primers. In comparison to other bacterial genera, homology was found only between the M HYOR FOR primer and most *Borrelia* species.

In addition, to ascertain the absence of cross-reactivity between samples, the separate primer couples were tested in single PCRs. Twenty-five cycles (30 s at 94°C; 15 s at 54°C; and 1 min at 72°C) were run on a GeneAmp 9600 Thermal Cycler (Perkin Elmer) using 2.5 U recombinant *Taq* DNA polymerase (Invitrogen), 1 × Taq buffer, 75 nmol $MgCl_2$, 10 nmol of each dNTP, and 10 pmol of one of the forward primers as well as the reverse primer. For each primer couple, 10 ng genomic template DNA of *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*, respectively, was tested in separate tubes.

Sensitivity

The concentration of genomic DNA of the different *Mycoplasma* species was determined by measuring OD_{260}. A 10-fold serial dilution of the genomic DNA was made and the different dilutions were tested for their reaction in the multiplex PCR. The minimal dilution still positive in the multiplex reaction was further diluted 2-fold. The minimum concentration still showing a positive result was noted.

RESULTS

Multiplex PCR

The multiplex reaction on purified genomic DNA of the three *Mycoplasma* species generated the expected bands, which were clearly distinguishable on a 1% agarose gel (Figure 1, lanes 1–3). When using the DNA mix, all three expected bands were observed (Figure 1, lane 5), while no band was observed with *M. hyosynoviae* (Figure 1, lane 4).

Sample preparation method

The multiplex PCR carried out directly on growing cultures or on the boiled mixture gave a negative result. The use of proteinase K during sample preparation had a positive effect, since all reactions resulted in the correct PCR product, although the intensity was often low and varied between different samples. A good, clear amplification product was obtained for all 15 tested isolates with the fourth preparation method using the alkaline lysis buffer (data not shown).

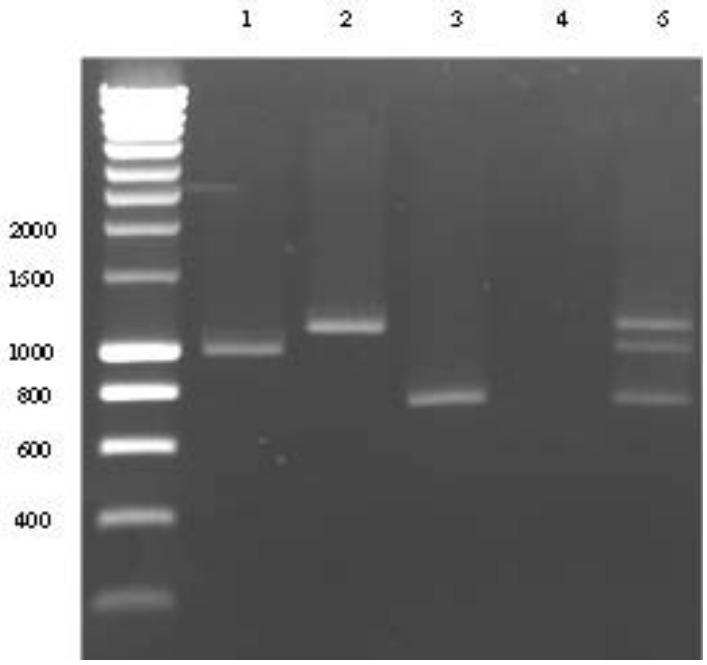

Figure 1. Multiplex reaction with DNA of *M. hyopneumoniae* (lane 1), *M. hyorhinis* (lane 2), *M. flocculare* (lane 3), *M. hyosynoviae* (negative control, lane 4), and with mixed DNA of the first three species (lane 5). The SmartLadder (Eurogentec, Seraing, Belgium) was used as size marker

Specificity

The three single PCR reactions provided the expected bands of 1129 bp (*M. hyorhinis*), 1000 bp (*M. hyopneumoniae*) and 754 bp (*M. flocculare*), while non-specific bands were absent (Figure 2). As expected, no PCR product was generated using genomic DNA of *M. hyosynoviae*.

Sensitivity

Using purified DNA, as little as 500 fg genomic DNA of *M. hyorhinis* and 1 pg genomic DNA of *M. hyopneumoniae* and *M. flocculare* could be detected (Figure 3).

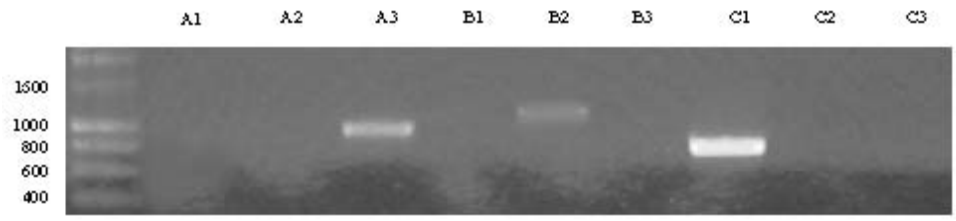

Figure 2. A PCR reaction was carried out with primers M REV and M HYOP FOR (lanes A), M HYOR FOR (lanes B), and M FLOC FOR (lanes C), respectively. Purified DNA of *M. flocculare* Ms42 strain (lanes 1), *M. hyorhinis* BTS-7 reference strain (lanes 2), and *M. hyopneumoniae* J-strain (lanes 3) was used as DNA template. The SmartLadder (Eurogentec) was used as size marker

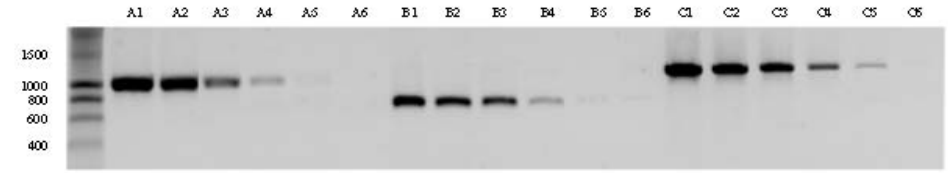

Figure 3. Detection limit of the multiplex PCR for *M. hyopneumoniae* (A), *M. flocculare* (B), and *M. hyosynoviae* (C) performed with 1 ng (lanes 1), 100 pg (lanes 2), 10 pg (lanes 3), 1 pg (lanes 4), 0.5 pg (lanes 5) and 0.25 pg (lanes 6) of purified DNA. The SmartLadder (Eurogentec) was used as size marker and the picture was inverted for reasons of clarity

DISCUSSION

M. hyopneumoniae, *M. flocculare* and *M. hyorhinis* are fastidious bacteria and are time-consuming to isolate from porcine lungs. Because they are able to grow in the same isolation medium, a fast and easy method to differentiate these strains would be a helpful tool during diagnosis. A multiplex PCR was accordingly developed. Three different forward primers were selected in a species-specific region, while the reverse primer was based on a highly conserved region of the 16S rRNA gene in mycoplasmas. The multiplex PCR may therefore be extended to other *Mycoplasma* spp. by choosing an appropriate forward primer.

Growing mycoplasma cultures were treated with alkaline lysis buffer before setting up the multiplex PCR. The importance of PCR-sample processing prior to the amplification reaction was reviewed recently (Radstrom *et al.*, 2004). The supplemented sera present in the isolation medium may have caused the observed inhibitory effect on the PCR reaction when working directly on broth culture (Al-Soud and Radstrom, 2001). The presence of these inhibitors may also explain the negative results obtained after boiling. Indeed, PCR inhibitors resistant to heat treatment have been reported before (Wiedbrauk *et al.*, 1995). Apparently, at least some of these inhibitors were proteins, since treatment with proteinase K produced the expected PCR fragments. The differences noted between different samples, the higher costs, as well as the long incubation period needed, makes this approach a less interesting alternative compared to the proposed method using alkaline lysis buffer.

The specificity of the multiplex PCR was tested using each forward primer together with the common reverse primer in separate PCRs. Another porcine *Mycoplasma* species, *M. hyosynoviae*, cannot be isolated using the same isolation medium (Friis, 1974), but since it is often found in lungs and tonsils of pigs (Friis *et al.*, 1991), it was included in the tests. No cross-reaction was noted. In addition, the primers showed no match with other mycoplasma sequences during our BLAST search. Only the M HYOR FOR primer matched the 16S rDNA of *Borrelia* spp. The presence of *Borrelia* spp. in lungs of pigs has, as far as we know, not been investigated. Even so, their growth would be inhibited by the antibiotics present in the media used (Johnson *et al.*, 1984).

Sensitivity testing proved the multiplex reaction to be very sensitive. Since only one copy of 16S rDNA is present in *M. hyopneumoniae* and *M. flocculare* (Taschke *et al.*, 1986) and given that the genomic size of one mycoplasma is approximately 1000 kilobase pairs, theoretically as little as 1000 microorganisms can be detected. This is close to the sensitivity of a PCR reaction for *M. hyopneumoniae* described by Blanchard and colleagues (1996). A much higher sensitivity, even on clinical samples, was attained by the use of nested PCR on 16S sequences of *M. hyopneumoniae* (Stärk *et al.*, 1998; Calsamiglia *et al.*, 1999b). Since our multiplex PCR is also based on 16S rDNA sequences, a similar detection limit might be expected using an extra amplification step. However, since we suggest a first isolation enrichment of the mycoplasmas, sensitivity is of much less concern.

The multiplex PCR generated species-specific amplicons that were easily distinguishable using standard gel electrophoresis. Because it is generally accepted that the more efficiently amplified loci negatively influence the yield of others, only one

PCR product is expected to be visible if one species is strongly dominating (Walsh *et al*., 1992). Nevertheless, simultaneous detection of similar amounts of different mycoplasmas was clearly shown.

In conclusion, the multiplex PCR can be used to detect and identify *M. hyopneumoniae*, *M. flocculare* and *M. hyorhinis*. To our knowledge, this is the first report to simultaneously differentiate these three *Mycoplasma* species potentially present in the lungs of pigs by means of a multiplex PCR.

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