# 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species

Laura McAuliffe,[1] Richard J. Ellis,[2] Jo R. Lawes,[1] Roger D. Ayling[1] and Robin A. J. Nicholas[1]

[1]Mycoplasma Group, Department of Statutory and Exotic Bacterial Diseases, Veterinary Laboratories Agency, Weybridge, Surrey KT15 3NB, UK

[2]NERC Centre for Population Biology, Imperial College London, Silwood Park Campus, Ascot, Berkshire SL5 7PY, UK

Correspondence
Laura McAuliffe
l.mcauliffe@vla.defra.gsi.gov.uk

Received 25 February 2005
Accepted 2 May 2005

Diagnosis of *Mycoplasma* infection is normally based on culture and serological tests, which can be time-consuming and laborious. A number of specific PCRs have been developed but to date there has not been a single generic test capable of detecting and differentiating mycoplasmas to a species level. This report describes the development of a new diagnostic test based on PCR of the 16S rRNA gene with *Mycoplasma*-specific primers and separation of the PCR product according to primary sequence using denaturing gradient gel electrophoresis (DGGE). DGGE enabled the differentiation of 67 *Mycoplasma* species of human and veterinary origin and represents a significant improvement on current tests as diagnosis of *Mycoplasma* infection can be made directly from clinical samples in less than 24 h.

## INTRODUCTION

Mycoplasmas cause a wide range of diseases in both humans and animals and are commonly associated with pneumonia, arthritis, conjunctivitis, infertility and abortion. Specific diagnosis of *Mycoplasma* infections is often difficult due to the limitations of current diagnostic tests together with the similarities in the diseases that they cause. *Mycoplasma* are highly fastidious; they typically take weeks to culture and many serological tests are non-specific and insensitive. More recently, PCR has been used to detect a number of *Mycoplasma* species. However, with over 102 mycoplasmas currently recognized it is not feasible to develop PCR tests for each species and there is a pressing need for a single generic test that can both detect and differentiate mycoplasmas.

Denaturing gradient gel electrophoresis (DGGE) can theoretically detect single-base mutations in DNA (Lerman & Beldjord, 1999; Fischer & Lerman, 1983). The method is based on the prevention of migration of DNA fragments following strand separation caused by chemical denaturants. DGGE has been used extensively for diversity analysis in microbial ecology (Muyzer, 1999) but has not been widely used for the identification and differentiation of pathogenic bacteria. Previously we demonstrated the ability of DGGE to detect and differentiate 27 mycoplasmas of veterinary importance using universal primers for the V3 region of 16S rDNA (McAuliffe *et al.*, 2003). The development of *Mycoplasma*-specific primers has enabled the application of this method directly to clinical material such as swabs and tissue samples. In addition, we have also extended the scope of the DGGE method to include human, equine, sea mammal, canine and feline *Mycoplasma* species and a variety of field isolates. The generic nature of the test may lead to the detection of *Mycoplasma* infections that would be difficult to identify using traditional culture techniques. The applicability of this method to mixed infections is also described.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; IGS, intergenic spacer.

## METHODS

**Strains and growth conditions.** The bacterial strains used in this study are listed in Tables 1 and 2. All strains were stored at −70 °C and grown at 37 °C with 5 % $CO_2$ without aeration. In addition to the type strains used in this study, a number of field strains were also tested using DGGE to ensure that there was intraspecific stability of DGGE profiles. Porcine mycoplasmas were grown in Friis broth and all other mycoplasmas were grown in Eaton's broth as previously described (Nicholas & Baker, 1998).

**Design of *Mycoplasma*-specific primers.** A specific reverse primer for *Mollicutes* was designed using Primrose (Ashelford *et al.*, 2002). A reverse primer, R543 (5′-ACCTATGTATTACCGCG), for *Mycoplasma* species was designed by aligning 102 *Mycoplasma* species. The forward primer of Muyzer *et al.* (1993), GC341, was used as described below. A 340 bp PCR product was generated with all 72 mycoplasmas tested. The

**Table 1.** Effect of annealing temperature on specificity of *Mycoplasma* 16S rDNA primers on a range of bacterial pathogens

| Organism and strain designation | PCR result at specific annealing temperature | | |
|---|---|---|---|
| | 55 °C | 56 °C | 57 °C |
| *Pseudomonas aeruginosa* NCTC 10332 | – | – | – |
| *Listeria monocytogenes* NCTC 10357 | + | – | – |
| *Bacillus cereus* NCTC 2599 | + | – | – |
| *Staphylococcus aureus* NCTC 8532 | + | – | – |
| *Haemophilus somnus* 129PT | – | – | – |
| *Streptococcus suis* NCTC 10234 | + | – | – |
| *Mycoplasma haemofelis* UK No. 5 | – | – | – |
| *Mycoplasma mycoides* subsp. *mycoides* SC NCTC 10114 | + | + | + |
| *Mycoplasma pneumoniae* NCTC 10119 | + | + | + |
| *Mycoplasma bovis* NCTC 10131 | + | + | + |
| *Mycoplasma hyopneumoniae* NCTC 10110 | + | + | + |
| *Acholeplasma laidlawii* NCTC 10116 | + | + | + |

**Table 2.** Use of DGGE directly on clinical samples

| Host species | No. of samples tested | Tissue type | No. positive by DGGE | *Mycoplasma* species detected by DGGE |
|---|---|---|---|---|
| Small ruminant | 56 | Eye swab | 2 | *Mycoplasma conjunctivae* |
| | | Lung | 6 | *Mycoplasma ovipneumoniae*, *Mycoplasma arginini* |
| | | Nasal swab | 21 | *Mycoplasma arginini, Mycoplasma ovipneumoniae*, *Mycoplasma conjunctivae* |
| | | Synovial joint fluid | 1 | *Mycoplasma ovipneumoniae* |
| | | Milk | 2 | *Mycoplasma ovipneumoniae* |
| Porcine | 55 | Lung | 12 | *Mycoplasma arginini*, *Mycoplasma hyorhinis* |
| Bovine | 39 | Lung | 6 | *Mycoplasma bovirhinis*, *Mycoplasma alkalescens*, *Mycoplasma arginini*, *Mycoplasma bovis.* |
| | | Nasal swab | 10 | *Mycoplasma bovirhinis*, *Mycoplasma bovigenitalium* |
| | | Bronchoalveolar lavage | 1 | *Mycoplasma bovirhinis* |
| | | Milk | 6 | *Mycoplasma bovis*, *Mycoplasma bovirhinis*, *Mycoplasma arginini* |
| | | Foetal stomach contents | 1 | *Mycoplasma bovirhinis* |
| | | Tracheal wash | 2 | *Mycoplasma bovirhinis* |
| | | Brain | 1 | *Mycoplasma bovis* |
| Avian | 52 | Eye swab | 13 | *Mycoplasma gallinarum*, *Mycoplasma gallinaceum*, *Mycoplasma iners*, |
| | | Sinus swab | 1 | *Mycoplasma pullorum* |
| | | Foot swab | 1 | *Mycoplasma cloacale* |
| | | Trachea | 3 | *Mycoplasma gallinaceum*, *Mycoplasma gallinarum* |

mollicute-specific reverse primer was tested against a range of other bacterial pathogens to ensure specificity as summarized in Table 1. A gradient thermocycler (Bio-Rad, iCycler) was used to test a range of annealing temperatures to ensure specificity. For all further experiments an annealing temperature of 56 °C was used.

**DNA extraction and 16S PCR.** *Mycoplasma* DNA was extracted from a 1 ml aliquot of stationary-phase culture using the Genelute genomic DNA kit according to the manufacturer's instructions (Sigma). DNA was extracted from swabs by swirling the swab in 1 ml of PBS, removing the swab and then using the Genelute kit as described above. DNA was extracted from tissue samples by removing a 1 $cm^2$ portion of tissue using sterile instruments, placing it in 1 ml of PBS, homogenizing to produce a suspension and extracting DNA using a Sigma tissue kit according to the manufacturer's instructions. Amplification of the V3 region of the 16S RNA gene was performed according to the method of Muyzer *et al.* (1993) with minor modifications using the universal bacterial primer GC-341F (5′-CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG) and the mollicute-specific primer R543. For the PCR, 1 µl lysate was added as a

template to 49 μl of a reaction mixture containing 10 mM Tris/HCl (pH 9·0), 1·5 mM $MgCl_2$, 50 mM KCl, 0·1 % Triton X-100, 0·2 mM of each deoxynucleoside triphosphate and 0·5 U Taqgold (Applied Biosystems). The cycling conditions were: denaturation at 94 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 56 °C for 45 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min, and samples were kept at 4 °C until analysis. Aliquots were checked for correct amplification by electrophoresis on a 2 % agarose gel followed by visualization with ethidium bromide under UV illumination.

**DGGE.** DGGE was performed using the Ingeny phorU 2×2 apparatus (GRI Molecular Biology). Samples (20 μl) were loaded onto 10 % polyacrylamide/bis (37·5 : 1) gels with denaturing gradients from 30–60 % [where 100 % is 7 M urea and 40 % (v/v) deionized formamide] in 1× TAE electrophoresis buffer (Severn Biotech). Electrophoresis was performed at 100 V at a temperature of 60 °C for 18 h. Gels were then stained with SBYR Gold (Cambridge BioScience) in 1× TAE for 30 min at room temperature and visualized under UV illumination.

## RESULTS

### *Mycoplasma*-specific primers

Members of *Mycoplasma*, *Acholeplasma* and *Ureaplasma* groups could be amplified using the *Mycoplasma*-specific primer R543 and the universal primer GC341; however, members of the related haemoplasma group could not. All 72 *Mycoplasma* species tested produced a PCR product of approximately 340 bp. Although some non-specific bands were found for *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* at 55 °C, increasing the annealing temperature to 56 °C ensured that the primers amplified only mollicute DNA (Table 1). These products were subjected to DGGE in groups according to host animal. In the majority of cases only one band was seen, indicating that there was no interspecific variation in the amplified sequence. The presence of multiple bands indicated that more than one 16S rDNA operon was present and that there were some sequence differences between the copies. The migration of the bands in the gels is a function of the melting behaviour of the amplicons in the chemical gradient used. A faint background band was sometimes seen on the DGGE gels, it is likely that this is due to a degree of primer-dimer formation and should as such be considered an artefact. The background band was easily differentiated from bands generated from 16S operons as it was faint in intensity, had an irregular shape and was not straight.

### Applicability of DGGE directly to clinical samples

In order to test the practicality of DGGE in the clinical laboratory, DNA extraction was performed directly on swabs and tissue samples received for veterinary diagnostic investigations. In total 202 clinical samples were analysed, of which 89 were found to be positive for *Mycoplasma* infection. *Mycoplasma* DNA was successfully amplified for DGGE from a wide variety of diagnostic samples including nasal, eye, ear and foot swabs, lung tissue, milk, brain tissue, synovial joint fluid and tissue from an aborted bovine foetus (summarized in Table 2). In order to test the robustness of the procedure on samples that had undergone long-term storage, DGGE was used on bovine lung samples obtained from outbreaks of contagious bovine pleuropneumonia in Botswana and Tanzania that had been frozen at −80 °C for approximately 9 years. DGGE identified *Mycoplasma mycoides* subsp. *mycoides* small colony (SC) in eight out of nine samples; culture of the lung samples also yielded *M. m*. subsp. *mycoides* SC in eight out of nine samples.

### Use of DGGE to detect mixed cultures

DGGE using *Mycoplasma*-specific primers was particularly useful for detecting mixed cultures. As shown in Fig. 1, analysis of a number of bovine diagnostic samples demonstrated that a mixed infection of *Mycoplasma bovirhinis*/*Mycoplasma alkalescens* could be detected easily. In addition, analysis of small ruminant clinical samples showed that mixed infections of *Mycoplasma ovipneumoniae*/*Mycoplasma arginini*, *M. ovipneumoniae*/*Mycoplasma conjunctivae* and *M. conjunctivae*/*M. arginini* could be detected.

### Intraspecific stability of DGGE profiles

To test that DGGE profiles were stable within a *Mycoplasma* species, at least 15 field isolates were compared with the type strain for a number of common veterinary pathogens including *Mycoplasma bovis*, *Mycoplasma agalactiae*, *M. ovipneumoniae*, *Mycoplasma gallinarum*, *Mycoplasma gallinaceum* and *M. m*. subsp. *mycoides* SC. No intraspecific variability was seen for any *Mycoplasma* species tested, with

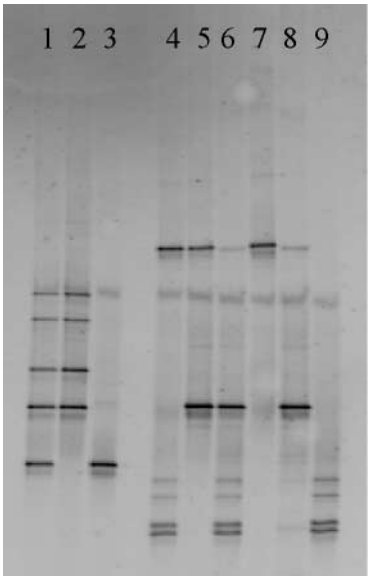


**Fig. 1**. Use of DGGE to detect mixed *Mycoplasma* infections in cattle and sheep: Lane 1, *M. bovirhinis* and *M. alkalescens* mixed field strains; lane 2, *M. bovirhinis* NCTC; lane 3, *M. alkalescens* NCTC; lane 4, *M. ovipneumoniae* and *M. arginini* mixed field strains; lane 5, *M. ovipneumoniae* and *M. conjunctivae* mixed field strains; lane 6, *M. conjunctivae* and *M. arginini* mixed field strains; lane 7, *M. ovipneumoniae* NCTC; lane 8, *M. conjunctivae* NCTC; lane 9, *M. arginini* NCTC.

the exception of *M. m*. subsp. *mycoides* SC and another member of the *Mycoplasma mycoides* cluster, *Mycoplasma capricolum* subsp. *capripneumoniae*. Most (23 of 24) of the *M. m*. subsp. *mycoides* SC strains tested gave an identical banding pattern of four bands on DGGE; however, the vaccine strain T144 gave a single band (results not shown). Analysis of *M. c*. subsp. *capripneumoniae* indicated some diversity of the 16S operons within the species. Two distinct profiles were seen: a profile identical to that of *Mycoplasma capricolum* subsp. *capricolum* was seen in three isolates and a profile of four bands that was distinct from all other profiles was seen for four other isolates (Fig. 2). There was some correlation between the geographical origin of the isolates and their DGGE profiles as isolates from Eritrea (strains T5, T6, T9 and T10) gave a distinctive profile unlike any other *Mycoplasma* species. Strain F38, which originated in Kenya, strain 44F04 from Turkey and strain 4/2 from Oman all gave identical profiles to *M. c*. subsp. *capricolum*.

## DGGE of human *Mycoplasma* species

All 11 human *Mycoplasma* species tested could be differentiated using DGGE (Fig. 3). *Mycoplasma primatum* and *Mycoplasma fermentans* had a similar migration pattern.

## DGGE of avian *Mycoplasma* species

Sixteen avian mycoplasmas could be easily distinguished using DGGE (summarized in Table 3). Perhaps most importantly, DGGE could distinguish the four avian *Mycoplasma* species of major economic importance, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* and *Mycoplasma iowae*. However, *M. iowae* and *Mycoplasma glycophilum* gave similar profiles, but when their full-length 16S sequences were compared, using a two-way BLAST alignment, only 80 % similarity was found (AF412981 *M. glycophilum* and M24293 *M. iowae*). Two pigeon *Mycoplasma* species could not be differentiated using DGGE. *Mycoplasma columborale* and *Mycoplasma columbinasale* could not be distinguished and gave the same profile by DGGE. However, analysis of the 16S–23S intergenic spacer (IGS) regions for *M. columborale* and *M. columbinasale* (AY796061 and AY796062, respectively) indicated that the species were not highly similar, with only 84 % congruence. BLAST of a shorter IGS on *M. columbinasale* AJ780986 indicated only 99/122 (81 %) similarity, with gaps of 12/122 (9 %).

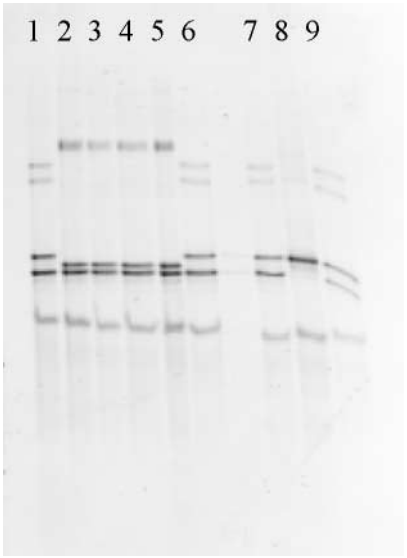


**Fig. 2.** Intraspecific variability of *M. c*. subsp. *capripneumoniae* isolates as shown by DGGE. Lane 1, *M. c*. subsp. *capripneumoniae* strain F38; lane 2, *M. c*. subsp. *capripneumoniae* strain T5; lane 3, *M. c*. subsp. *capripneumoniae* strain T6; lane 4, *M. c*. subsp. *capripneumoniae* strain T9; lane 5, *M. c*. subsp. *capripneumoniae* strain T10; lane 6, *M. c*. subsp. *capripneumoniae* strain 44F04; lane 7, *M. c*. subsp. *capripneumoniae* strain 4/2; lane 8, *M. m*. subsp. *mycoides* LC; lane 9, *M. c*. subsp. *capricolum*.

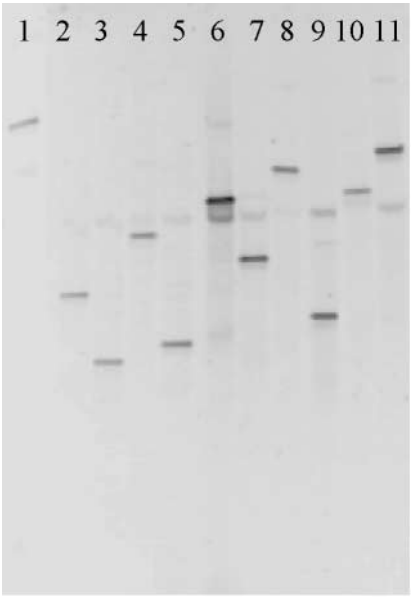


**Fig. 3.** DGGE of human *Mycoplasma* species. Lane 1, *Mycoplasma pneumoniae*; lane 2, *Mycoplasma hominis*; lane 3, *Mycoplasma faucium*; lane 4, *Mycoplasma buccale*; lane 5, *Mycoplasma arthritidis*; lane 6, *Mycoplasma spermatophilum*; lane 7, *Mycoplasma salivarum*; lane 8, *M. primatum*; lane 9, *Mycoplasma orale*; lane 10, *Mycoplasma genitalium*; lane 11, *M. fermentans*.

## Marine isolates

The sea mammal *Mycoplasma* species *Mycoplasma phocarhinis*, *Mycoplasma phocicerebrale* and *Mycoplasma phocidae* were easily distinguished using DGGE (Fig. 4). Interestingly, a feline mycoplasma, *Mycoplasma gateae*, gave an identical profile to *M. phocicerebrale* (Fig. 4). Comparison of DNA 16S–23S IGS sequences for *M. gateae* and *M. phocicerebrale* (AF443609 and AY766092, respectively) revealed a high degree of similarity between the two sequences, with similarity of 97 % and gaps of only 1 % as determined using a two-

**Table 3.** *Mycoplasma* strains, their origin and host, and a summary of DGGE results

| Species | Strain designation | Host species | Specific detection by DGGE/comments |
|---|---|---|---|
| *Mycoplasma anatis* | NCTC 10156 | Avian | + |
| *Mycoplasma cloacale* | NCTC 10199 | Avian | + |
| *Mycoplasma columbinasale* | NCTC 10184 | Avian | −; same as *M. columborale* |
| *Mycoplasma columbinum* | NCTC 10178 | Avian | + |
| *Mycoplasma columborale* | NCTC 10179 | Avian | −; same as *M. columbinasale* |
| *Mycoplasma gallinaceum* | NCTC 10183 | Avian | + |
| *Mycoplasma gallinarum* | NCTC 10120 | Avian | + |
| *Mycoplasma gallisepticum* | PG31T | Avian | + |
| *Mycoplasma gallopavonis* | NCTC 10186 | Avian | + |
| *Mycoplasma glycophilum* | NCTC 10194 | Avian | +; similar to *M. iowae* |
| *Mycoplasma imitans* | NCTC 11733 | Avian | + |
| *Mycoplasma iners* | NCTC 10165 | Avian | + |
| *Mycoplasma iowae* | NCTC 10185 | Avian | +; similar to *M. glycophilum* |
| *Mycoplasma lipofaciens* | NCTC 10191 | Avian | + |
| *Mycoplasma meleagridis* | NCTC 10153 | Avian | + |
| *Mycoplasma pullorum* | NCTC 10187 | Avian | + |
| *Mycoplasma synoviae* | NCTC 10124 | Avian | + |
| *Mycoplasma alkalescens* | NCTC 10135 | Bovine | + |
| *Mycoplasma alvi* | NCTC 10157 | Bovine | + |
| *Mycoplasma bovigenitalium* | NCTC 10122 | Bovine | +; same as *M.* species ovine/caprine serogroup 11 but both are likely to be assigned to the same species |
| *Mycoplasma species* bovine group 7 | NCTC 10133 | Bovine | + |
| *Mycoplasma bovirhinis* | NCTC 10118 | Bovine | + |
| *Mycoplasma bovis* | NCTC 10131 | Bovine | +; similar to *M. verecundum* and *M. canadense* |
| *Mycoplasma bovoculi* | NCTC 10141 | Bovine | + |
| *Mycoplasma californicum* | NCTC 10189 | Bovine | + |
| *Mycoplasma canadense* | NCTC 10152 | Bovine | +; similar to *M. verecundum* and *M. bovis* |
| *Mycoplasma canis* | NCTC 10146 | Bovine/canine | +; different to all bovine species but same as *M. edwardii* |
| *Mycoplasma dispar* | NCTC 10125 | Bovine | + |
| *Mycoplasma mycoides* subsp. *mycoides* small-colony type | PG1 | Bovine | + |
| *Mycoplasma verecundum* | NCTC 10145 | Bovine | +; similar to *M. canadense* and *M. bovis* |
| *Mycoplasma cynos* | NCTC 10142 | Canine | + |
| *Mycoplasma edwardii* | NCTC 10132 | Canine | −; same as *M. canis* |
| *Mycoplasma maculosum* | NCTC 10168 | Canine | +; same as *M. leopharyngis* |
| *Mycoplasma opalescens* | NCTC 10149 | Canine | + |
| *Mycoplasma spumans* | NCTC 10169 | Canine | + |
| *Mycoplasma equigenitalium* | ATCC 29869 | Equine | + |
| *Mycoplasma equirhinis* | NCTC 10148 | Equine | + |
| *Mycoplasma fastidiosum* | NCTC 10180 | Equine | + |
| *Mycoplasma felis* | NCTC 10160 | Equine | + |
| *Mycoplasma subdolum* | NCTC 10175 | Equine | + |
| *Mycoplasma arthritidis* | NCTC 10162 | Human | + |
| *Mycoplasma buccale* | NCTC 10136 | Human | + |
| *Mycoplasma faucium* | NCTC 10174 | Human | + |
| *Mycoplasma fermentans* | NCTC 10117 | Human | +; similar to *M. primatum* |
| *Mycoplasma genitalium* | NCTC 10195 | Human | + |
| *Mycoplasma hominis* | NCTC 10111 | Human | + |
| *Mycoplasma lipophilum* | NCTC 10173 | Human | + |
| *Mycoplasma orale* | NCTC 10112 | Human | + |
| *Mycoplasma pneumoniae* | NCTC 10119 | Human | + |

**Table 3.** cont.

| Species | Strain designation | Host species | Specific detection by DGGE/comments |
|---|---|---|---|
| *Mycoplasma primatum* | NCTC 10163 | Human | +; similar to *M. fermentans* |
| *Mycoplasma salivarum* | NCTC 10113 | Human | + |
| *Mycoplasma spermatophilum* | NCTC 11720 | Human | + |
| *Mycoplasma flocculare* | NCTC 10143 | Porcine | + |
| *Mycoplasma hyopneumoniae* | NCTC 10110 | Porcine | + |
| *Mycoplasma hyorhinis* | NCTC 10130 | Porcine | + |
| *Mycoplasma hyosynoviae* | NCTC 10167 | Porcine | + |
| *Mycoplasma gateae* | NCTC 10161 | Sea mammal/feline | −; same as *M. phocicerebrale* |
| *Mycoplasma phocicerebrale* | NCTC 11721 | Sea mammal | −; same as *M. gateae* |
| *Mycoplasma phocidae* | Strain 105 | Sea mammal | + |
| *Mycoplasma phocirhinis* | NCTC 11722 | Sea mammal | + |
| *Mycoplasma agalactiae* | NCTC 10123 | Small ruminant | + |
| *Mycoplasma arginini* | NCTC 10129 | Small ruminant | + |
| *Mycoplasma conjunctivae* | NCTC 10147 | Small ruminant | + |
| *Mycoplasma cottewii* | NCTC 11732 | Small ruminant | −; same as *M. yeatsii* |
| *Mycoplasma ovipneumoniae* | NCTC 10151 | Small ruminant | + |
| *Mycoplasma* ovine/caprine serogroup 11 | Strain 2D | Small ruminant | +; same as *M. bovigenitalium* but both are likely to be assigned to the same species |
| *Mycoplasma putrefaciens* | NCTC 10155 | Small ruminant | + |
| *Mycoplasma capricolum* subsp. *capricolum* | NCTC 10154 | Small ruminant | + |
| *Mycoplasma capricolum* subsp. *capripneumoniae* | NCTC 10192 | Small ruminant | + |
| *Mycoplasma mycoides* subsp. *capri* | NCTC 10137 | Small ruminant | +; indistinguishable from *M. m*. subsp. *mycoides* LC but both are likely to be the same species |
| *Mycoplasma mycoides* subsp. *mycoides* large-colony type | F30 | Small ruminant | +; indistinguishable from *M. m*. subsp. *capri* but both are likely to be the same species |
| *Mycoplasma yeatsii* | NCTC 11730 | Small ruminant | −; same as *M. cottewii* |

way BLAST alignment (bl2seq, NCBI). Comparison of full-length 16S sequences also revealed congruence between the sequences, with 98 % similarity and no gaps (U15796 and AF304323 for *M. gateae* and *M. phocicerebrale*, respectively).

## Bovine *Mycoplasma* species

DGGE could differentiate all 13 bovine *Mycoplasma* species tested (as summarized in Table 3). A similar migration pattern was seen in three bovine species, *Mycoplasma verecundum*, *Mycoplasma canadense* and *M. bovis*. However, careful analysis showed that there was a small difference in the distance of migration between the three species. *M. bovis* produced a different profile to that of the small ruminant mycoplasma *M. agalactiae*, which can be difficult to distinguish from *M. bovis* by normal culture and serological tests. Significantly, *M. m*. subsp. *mycoides* SC, the causative agent of contagious bovine pleuropneumonia (CBPP) was easily distinguished from all other *Mycoplasma* species tested and had a characteristic pattern of four bands. *M. m*. subsp. *mycoides* SC was also easily distinguished from all other members of the closely related *M. mycoides* cluster.

## Small ruminant *Mycoplasma* species

Twelve small ruminant *Mycoplasma* species were analysed using DGGE (summarized in Table 3). All species gave easily distinguishable profiles except for the closely related *M. m*. subsp. *mycoides* large colony (LC) and *Mycoplasma mycoides* subsp. *capri*, which were identical; similarly, *M. cottewii* and *M. yeatsii* could not be differentiated. Analysis of full-length 16S sequences and 16S–23S spacer of *M. m*. subsp. *mycoides* LC and *M. m*. subsp. *capri* showed a very high degree of similarity (>99 %) between the species, in line with previous studies that have suggested that the two species should be amalgamated into a single species (Pettersson *et al.*, 1996). Similarly *Mycoplasma yeatsii* and *Mycoplasma cottewii* were also at least 99 % similar when both full-length 16S and 16S–23S IGS were compared. Significantly, a number of members of the closely related *M. mycoides* cluster could be differentiated, and *Mycoplasma putrefaciens* gave a unique profile.

## Canine *Mycoplasma* species

The canine *Mycoplasma* species *Mycoplasma spumans*, *Mycoplasma opalescens*, *Mycoplasma cynos* and *Mycoplasma*

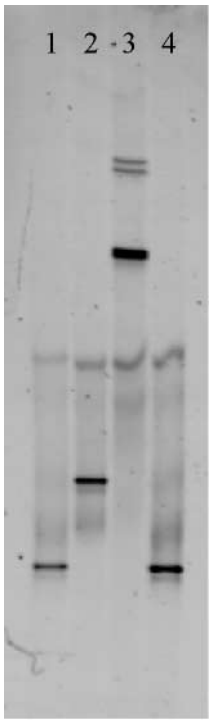


**Fig. 4.** DGGE of sea mammal *Mycoplasma* species. Lane 1, *M. phocicerebrale*; lane 2, *M. phocirhinis*; lane 3, *M. phocidae*; lane 4, *M. gateae*.

*maculosum* were easily distinguished using DGGE (Fig. 5). However, *Mycoplasma canis* and *Mycoplasma edwardii* gave highly similar profiles using DGGE; given the high 16S sequence homology between these two species (98 % with no gaps; U73903 and AF412972) this is not unexpected. Interestingly, when *M. maculosum* was compared with a number of feline isolates, it gave an identical profile to the lion mycoplasma *Mycoplasma leopharyngis*. Comparison of 16S and 16S–23S IGS sequences for *M. maculosum* and *M. leopharyngis* also indicated that the species are identical.

## Equine *Mycoplasma* species

The four main *Mycoplasma* species found in horses, *Mycoplasma subdolum*, *Mycoplasma fastidiosum*, *Mycoplasma equirhinis* and *Mycoplasma equigenitalium*, were all easily distinguishable using DGGE (Fig. 6). In addition the feline *Mycoplasma* species *Mycoplasma felis*, which has been associated with respiratory disease in horses (Ogilvie *et al*., 1983), was also easy to distinguish from the other equine-associated mycoplasmas using DGGE.

## Porcine *Mycoplasma* species

The four main porcine *Mycoplasma* species, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae* and *Mycoplasma flocculare*, were easily distinguished using DGGE (summarized in Table 3).

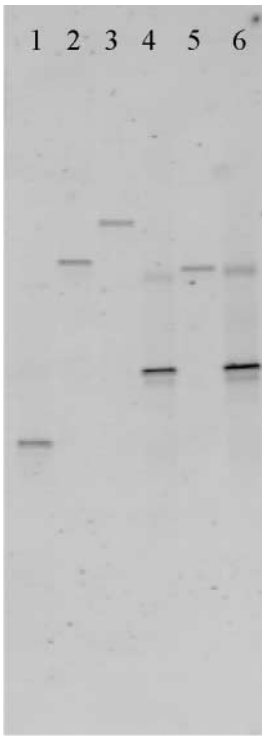


**Fig. 5.** DGGE of canine *Mycoplasma* species. Lane 1, *M. spumans*; lane 2, *M. opalescens*; lane 3, *M. maculosum*; lane 4, *M. edwardii*; lane 5, *M. cynos*; lane 6, *M. canis*.

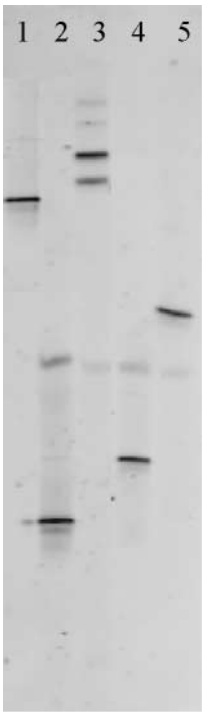


**Fig. 6.** DGGE of equine *Mycoplasma* species. Lane 1, *M. fastidiosum*; lane 2, *M. subdolum*; lane 3, *M. felis*; lane 4, *M. equirhinis*; lane 5, *M. equigenitalium*.

## DISCUSSION

DGGE analysis has enabled the detection and differentiation of 67 *Mycoplasma* species. For at least 40 of these *Mycoplasma* species there has not previously been a DNA-based diagnostic test available and many have only been identifiable through lengthy culture or serological tests. Previously we showed that DGGE could be used to differentiate 27 *Mycoplasma* species of veterinary importance (McAuliffe *et al.*, 2003). The current work extends that study to include 67 *Mycoplasma* species and presents significant improvements to the technique including the use of *Mycoplasma*-specific primers. Whereas DGGE using universal primers required a media-enrichment step to ensure that only mollicute DNA was amplified (McAuliffe *et al.*, 2003), with the advent of mollicute-specific primers, DGGE can be applied directly to clinical material. As a result of this, *Mycoplasma* infections can now be diagnosed in less than 24 h compared with 1–2 weeks for traditional culture. The use of *Mycoplasma*-specific primers has also enabled the detection of mixed cultures, which would have been difficult to detect by conventional methods, as less fastidious species would be outgrown.

DGGE may prove to be particularly useful for human mycoplasmas and is the first generic test capable of differentiating 11 species. Previously a multiplex PCR has been used to differentiate genital *Mycoplasma* species (Stellrecht *et al.*, 2004) and a reverse line blotting procedure has been used to differentiate five human mollicute pathogens (Wang *et al.*, 2004) but there has not been a single, generic test for other human *Mycoplasma* species.

Significantly *Mycoplasma genitalium* and *Mycoplasma pneumoniae* can be differentiated easily by DGGE, thus demonstrating the specificity of the technique as there is 98 % similarity between the two species based on 16S rDNA sequence homology (Jensen *et al.*, 2003).

A number of mycoplasmas could not be differentiated using DGGE and gave identical profiles. For example, *M. m.* subsp. *capri* and *M. m.* subsp. *mycoides* LC were indistinguishable, indicating that there was no variation in the 16S rDNA sequence over the V3 region amplified. This may provide further support for the notion that *M. m.* subsp. *mycoides* LC and *M. m.* subsp. *capri* are in fact the same species (Pettersson *et al.*, 1996).

Some unexpected isolates also gave identical profiles by DGGE, for example the feline mycoplasma *M. gateae* and the sea mammal species *M. phocicerebrale*. These results were also supported by comparison of full-length 16S and 16S–23S IGS sequences for the isolates, which also indicated a very high degree of similarity between the species. If these species are closely related it is difficult to explain how they could have been transmitted between two very different hosts, cats and seals, which seem unlikely to have come into close contact with one another. Similarly, the canine mycoplasma *M. maculosum* showed a high degree of similarity to the lion mycoplasma *M. leopharyngis* by 16S and 16S–23S IGS analysis and gave identical DGGE profiles. Previous studies have also highlighted the high degree of similarity in 16S sequence and identical biochemical characteristics of these species (Pettersson *et al.*, 2001).

Two canine *Mycoplasma* species, *M. canis* and *M. edwardii*, gave indistinguishable DGGE profiles. This is not unexpected as previous analysis of full-length 16S sequences and 16S–23S IGS sequences found that the species are highly similar (Chalker & Brownlie, 2004). Interestingly, *M. cynos* could be differentiated from all other canine *Mycoplasma* species whereas previous studies based on sequence analysis have shown it grouped closely with *M. canis* and *M. edwardii* (Chalker & Brownlie, 2004).

Two species, *Mycoplasma columbinum* and *M. columbinasale*, could not be distinguished, although previous studies have indicated that they are less than 97 % similar by 16S sequence analysis (Pettersson *et al.*, 2001). Even when cultures were obtained from several different collections the two isolates gave identical profiles. It is likely that the species were previously identified using serological tests, which emphasizes the need for DNA sequencing of historical isolates in collections to ensure that they are correctly identified. Although, whether species should be designated based on serological or molecular methods is still a contentious issue within *Mollicutes* taxonomy.

DGGE also showed potential for use in molecular-typing studies. Some intraspecific variation in 16S sequences was seen for members of the *M. mycoides* cluster, *M. m.* subsp. *mycoides* SC and *M. c.* subsp. *capripneumoniae*. There was some correlation between the origin of the isolates and the profiles obtained for *M. c.* subsp. *capripneumoniae* as isolates from Eritrea gave a distinct profile compared with those from Kenya, Turkey and Oman. Previous studies have found that sequencing of the 16S operons of *M. c.* subsp. *capripneumoniae* can be a useful tool for epidemiological analysis (Heldtander *et al.*, 2001). DGGE may enable rapid typing of strains and entails much simpler analysis compared with DNA sequencing.

Recently, denaturing HPLC analysis has been used to detect and type bacterial pathogens (Domann *et al.*, 2003; Hurtle *et al.*, 2003) and could theoretically be used as an alternative to DGGE to target single nucleotide polymorphisms in the V3 region of 16S rDNA of *Mycoplasma* species. However, denaturing-HPLC would require expensive, specialized equipment and more laborious standardization and interpretation compared with DGGE.

In conclusion, DGGE enables the rapid detection and differentiation of *Mycoplasma* species and can be used to diagnose infections either directly from tissues or from cultured isolates. It is capable of detecting mixed cultures or even new *Mollicutes* species and is suitable for routine use in the diagnostic laboratory.

## ACKNOWLEDGEMENTS

We wish to thank Defra for their continuing support, and Dr Séverine Tasker for the donation of Haemoplasma DNA.

## REFERENCES


**Ashelford, K. E., Weightman, A. J. & Fry, J. C. (2002).** PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Res* **30**, 3481–3489.

**Chalker, V. J. & Brownlie, J. (2004).** Taxonomy of the canine *Mollicutes* by 16S rRNA gene and 16S/23S rRNA intergenic spacer region sequence comparison. *Int J Syst Evol Microbiol* **54**, 537–542.

**Domann, E., Hong, G., Imirzalioglu, C., Turschner, S., Kuhle, J., Watzel, C., Hain, T., Hossain, H. & Chakraborty, T. (2003).** Culture-independent identification of pathogenic bacteria and polymicrobial infections in the genitourinary tract of renal transplant recipients. *J Clin Microbiol* **41**, 5500–5510.

**Fischer, S. G. & Lerman, L. S. (1983).** DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with theory. *Proc Natl Acad Sci U S A* **80**, 1579–1583.

**Heldtander, M., Wesonga, H., Bolske, G., Pettersson, B. & Johansson, K. E. (2001).** Genetic diversity and evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains from eastern Africa assessed by 16S rDNA sequence analysis. *Vet Microbiol* **78**,13–28.

**Hurtle, W., Bode, E., Kaplan, R. S., Garrison, J., Kearney, B., Shoemaker, D., Henchal, E. & Norwood, D. (2003).** Use of denaturing high-performance liquid chromatography to identify *Bacillus anthracis* by analysis of the 16S-23S rRNA interspacer region and *gyrA* gene. *J Clin Microbiol* **41**, 4758–4766.

**Jensen, J. S., Borre, M. B. & Dohn, B. (2003).** Detection of *Mycoplasma genitalium* by PCR amplification of the 16S rRNA gene. *J Clin Microbiol* **41**, 261–266.

**Lerman, L. S. & Beldjord, C. (1999).** Comprehensive mutation detection with denaturing gradient gel electrophoresis. In *Mutation Detection*, pp. 35–61. Edited by R. G. H. Cotton, E. Edkins and S. Forrest. New York: Oxford University Press.

**McAuliffe, L., Ellis, R. J., Ayling, R. D. & Nicholas, R. A. (2003).** Differentiation of *Mycoplasma* species by 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting. *J Clin Microbiol* **41**, 4844–4847.

**Muyzer, G. (1999).** DGGE/TGGE: a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* **2**, 317–322.

**Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993).** Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.

**Nicholas, R. & Baker, S. (1998).** Recovery of mycoplasmas from animals. *Methods Mol Biol* **104**, 37–43.

**Ogilvie, T. H., Rosendal, S., Blackwell, T. E., Rostkowski, C. M., Julian, R. J. & Ruhnke, L. (1983).** *Mycoplasma felis* as a cause of pleuritis in horses. *J Am Vet Med Assoc* **182**, 1374–1376.

**Pettersson, B., Leitner, T., Ronaghi, M., Bolske, G., Uhlen, M. & Johansson, K. E. (1996).** Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. *J Bacteriol* **178**, 4131–4142.

**Pettersson, B., Tully, J. G., Bolske, G. & Johansson, K. E. (2001).** Re-evaluation of the classical *Mycoplasma lipophilum* cluster (Weisburg *et al*. 1989) and description of two new clusters in the hominis group based on 16S rDNA sequences. *Int J Syst Evol Microbiol* **51**, 633–643.

**Stellrecht, K. A., Woron, A. M., Mishrik, N. G. & Venezia, R. A. (2004).** Comparison of multiplex PCR assay with culture for detection of genital mycoplasmas. *J Clin Microbiol* **42**, 1528–1533.

**Wang, H., Kong, F., Jelfs, P., James, G. & Gilbert, G. L. (2004).** Simultaneous detection and identification of common cell culture contaminant and pathogenic mollicutes strains by reverse line blot hybridization. *Appl Environ Microbiol* **70**, 1483–1486.