Tools for Detection of Mycoplasma amphoriforme: a Primary Respiratory Pathogen?

Clare L. Ling,a,* Katarina Oravcova,b Thomas F. Beattie,ab,c Dean D. Creer,d Paul Dilworth,a Naomi L. Fulton,f Alison Hardie,f Michelle Munro,f Marcus Pond,g Kate Templeton,b David Webster,b Sarita Workman,b Timothy D. McHugh,b Stephen H. Gillespieb

Centre for Clinical Microbiology, Department of Infection, Royal Free Campus, University College London, London, United Kingdom,a; School of Medicine, University of St. Andrews, St. Andrews, United Kingdomb; Royal Hospital for Sick Children, Edinburgh, United Kingdomc; Respiratory Medicine, Barnet General Hospital, London, United Kingdomd; University College London Medical School, London, United Kingdom,e; Department of Medical Microbiology, Royal Infirmary, Edinburgh, United Kingdomf; Centre for Infection and Immunity, St. George’s University of London, London, United Kingdomg; Department of Immunology, Royal Free London NHS Foundation Trust, London, United Kingdomh

Mycoplasma amphoriforme is a recently described organism isolated from the respiratory tracts of patients with immunodeficiency and evidence of chronic infection. Novel assays for the molecular detection of the organism by real-time quantitative PCRs (qPCRs) targeting the uracil DNA glycosylase gene (udg) or the 23S rRNA gene are described here. The analytical sensitivities are similar to the existing conventional M. amphoriforme 16S rRNA gene PCR, with the advantage of being species specific, rapid, and quantitative. By using these techniques, we demonstrate the presence of this organism in 17 (19.3%) primary antibody-deficient (PAD) patients, 4 (5%) adults with lower respiratory tract infection, 1 (2.6%) sputum sample from a patient attending a chest clinic, and 23 (0.21%) samples submitted for viral diagnosis of respiratory infection, but not in normal adult control subjects. These data show the presence of this microorganism in respiratory patients and suggest that M. amphoriforme may infect both immunocompetent and immunocompromised people. Further studies to characterize this organism are required, and this report provides the tools that may be used by other research groups to investigate its pathogenic potential.

Mycoplasma amphoriforme was first isolated in 1999 from a patient with primary antibody deficiency (PAD) with chronic bronchitis. It has also been isolated subsequently from both immunocompromised and immunocompetent patients with respiratory tract infections (RTI) in London, Denmark, France, and Tunisia (1–3). Based on 16S rRNA sequencing, M. amphoriforme belongs to the same phylogenetic group as other human pathogenic Mycoplasma species, the pneumoniae group (1, 2). The closest species phylogenetically for which there is a whole-genome sequence is Mycoplasma gallisepticum, a bird pathogen. Phenotypic studies have demonstrated that M. amphoriforme has features in common with this group, including gliding motility, a protruding polar tip resembling that of M. gallisepticum, and a cytoskeletal structure at its polar tip with homology to that of M. pneumoniae’s attachment organelle (1, 4).

To understand the role that this novel agent plays in human health, better laboratory tools are required. M. amphoriforme is fastidious, requiring specialized media for cultivation, and it takes approximately 2 weeks for colonies to appear on agar. The colonial morphology resembles granular droplets, making detection difficult, as the droplets can blend into the sample matrix and be overlooked. This paper reports the development and evaluation of two real-time quantitative PCR (qPCR) assays: one assay targeting M. amphoriforme’s uracil DNA glycosylase gene (udg) and one assay targeting the variable region of the 23S rRNA gene that is unique to M. amphoriforme. The new qPCR assays were compared with a previously reported 16S rRNA gene assay (2) and used to test a range of human samples from the United Kingdom.

MATERIALS AND METHODS

Patients, samples, and ethical approval. Clinical samples from two hospitals were used in this study: from the Royal Free London NHS Foundation Trust (RFL), Hampstead, and from the Royal Infirmary of Edinburgh (RIE). Approvals were obtained from the Ethics Committee of the RFL Hampstead and from the Lothian Regional Ethics Committee (08/S11/02/2) to retain information during the steps taken to retain anonymity for epidemiological purposes.

From 19 October 2000 to 6 September 2005, sputum samples were collected from PAD patients attending the dedicated Primary Immunodeficiency Clinic at the RFL. These patients attended the clinic for either a routine appointment or in cases of clinical deterioration. A sputum sample was collected from any patient with a productive cough and sent for microbiological investigation, including detection of Mycoplasma amphoriforme. The age range of all the PAD patients tested was 18 to 79 years, with an average age of 44 years.

Sputum and/or throat swab samples were collected from adult patients attending the RFL Chest Clinic and from adult patients (>18 years old) with lower respiratory tract infection (LRTI) who were recruited from two general practices with multiethnic patient populations (in total) of 15,000 and from social classes I to V, as described previously (5). All LRTI patients were surgery attendees; no recruitment was undertaken outside of clinic hours or on home visits. Acute LRTI was defined as a new or worsening cough and at least one other lower respiratory tract symptom for which there was no other explanation, present for 21 days or less (6, 7). Patients were excluded if they had underlying chronic supplicative
TABLE 1 Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide (5′–3′)*</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>udg</td>
<td>MAudgF, TGCGGCCGATAAAAACCGAATAT</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>MAudgR, TTTGCAAAAGGGTTTGTCAACAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe, FAM-TTGGTGCATCTCCTCACCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTAAGTGCGA-BHQ1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23S rRNA</td>
<td>Forward, GGGGTCATAATAACAGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse, CGGTATATGGCCTGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S rRNA</td>
<td>Ampf, AAGCTAGTAAAAGGAAATGTATT</td>
</tr>
<tr>
<td></td>
<td>Ampf-rt, ACTATAGAATAATAGTGC</td>
<td></td>
</tr>
</tbody>
</table>

*FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1.

Extraction of DNA. DNA was extracted from control organisms by using the Wizard genomic DNA extraction kit (Promega, Southampton, United Kingdom) following the manufacturer’s instructions. Extraction of DNA from sputum and throat swab samples was performed by using a Chelex-based extraction method; following centrifugation at 13,000 × g for 10 min, resulting pellets were washed three times with sterile phosphate-buffered saline (PBS), resuspended with PCR-grade water (5× the pellet volume), and vortexed with 10% Chelex (Sigma, Poole, United Kingdom) at a ratio of 1:1. After incubation at 56°C for 30 min followed by 94°C for 5 min, the samples were vortexed, cooled on ice, and then centrifuged at 13,000 × g for 2 min, and the resulting supernatants were used for PCR.

The DNA from respiratory samples for respiratory virus screening was extracted using the easyMAG system (bioMérieux, United Kingdom) and eluted into 100-μl volumes. All extracts were stored at −20°C until used.

M. amphiriforme 16S rDNA gene PCR. All oligonucleotides used in this study are listed in Table 1. The DNA extracts from all patient groups were tested for the presence of M. amphiriforme via the 16S rDNA PCR as previously described (2). The identity of the amplicons from at least the first positive sample from each patient was confirmed by sequencing using standard Sanger sequencing protocols. The sequences were analyzed using BioNumerics software version 3.5 (Applied Maths) and aligned using the CLUSTAL W multiple sequence alignment program (8). The consensus sequences were compared to the 16S rDNA gene sequence from the preliminary contiguous M. amphiriforme strain A39 whole-genome sequence obtained from the Wellcome Trust Sanger Institute.

M. amphiriforme qPCR. The oligonucleotides for the M. amphiriforme udg quantitative real-time PCR (Table 1) were designed and optimized. The optimized M. amphiriforme qPCR protocol consisted of 5 μl of template DNA, 1× Invitrogen Platinum QPCR SuperMix-UDG, 7 mM MgCl₂, 0.3 μM primer MAudgF, 0.9 μM primer MAudgR, and 0.25 μM probe MAudgP, in a final volume of 25 μl. The reactions were performed in a RotorGene 3000 apparatus (Qiagen, United Kingdom) under cycling conditions of 95°C for 3 min followed by 35 cycles of 95°C for 15 s and 58°C for 60 s. Results were analyzed with the cycle threshold set at 0.03. The standard curves were constructed in two independent experiments on serial 10-fold dilutions of M. amphiriforme DNA in triplicate samples.

The specificity of the assay was confirmed by the amplification of 1 ng of DNA from the control organisms listed above, in duplicate. The identities of amplicons were confirmed by Sanger sequencing.

The udg qPCR was performed on the DNA extracts from patients with PAD. Samples were tested neat, diluted 1:10, and spiked (4 μl of sample and 1 pg/μl M. amphiriforme DNA, to detect sample inhibition). Samples positive both neat and at a 10-fold DNA dilution were considered positive, and samples that were only positive either neat or at a 10-fold dilution were considered equivocal. To avoid bias due to sample storage, all samples with discrepant results were retested using the 16S rDNA PCR.

A quantitative real-time PCR assay targeting the variable region of the 23S rDNA gene unique for M. amphiriforme was designed and optimized.
confirmed by sequencing.

To further confirm the specificity of the assays, the identities of the amplicons were confirmed by sequencing, and the sequences were submitted to GenBank. None of the control samples (49 throat swabs from healthy individuals) were positive. All four M. amphoriforme-positive samples were taken from patients with clinical signs of acute LRTI, including raised pulse rate, respiratory rate, and C-reactive protein concentration compared with the controls. None had a history of recent travel, alcohol consumption, or steroid treatment. Other respiratory organisms were detected in two of the M. amphoriforme-positive patients: coronavirus, human rhinovirus, H. influenzae plus Streptococcus pneumoniae in the patient with M. amphoriforme-positive throat swab, and enterovirus in a patient with M. amphoriforme-positive sputum.

M. amphoriforme in patients with suspected LRTI. M. amphoriforme was detected in 4 (5%) out of 80 patients with LRTI (who had been recruited from general practices): 1/80 throat swabs and 3/50 sputum samples were positive by 16S rRNA PCR (Table 2). The identities of all PCR-positive amplicons were confirmed by sequencing, and the sequences were submitted to GenBank. None of the control samples (49 throat swabs from healthy individuals) were positive. All four M. amphoriforme-positive samples were taken from patients with clinical signs of acute LRTI, including raised pulse rate, respiratory rate, and C-reactive protein concentration compared with the controls. None had a history of recent travel, alcohol consumption, or steroid treatment. Other respiratory organisms were detected in two of the M. amphoriforme-positive patients: coronavirus, human rhinovirus, H. influenzae plus Streptococcus pneumoniae in the patient with M. amphoriforme-positive throat swab, and enterovirus in a patient with M. amphoriforme-positive sputum.

M. amphoriforme in patients with suspected respiratory viral infection. The respiratory samples screened for suspected LRTI used in this study were collected between 1 March 2011 and 11 March 2012. Out of 10,747 (3,496 from adults and 7,251 from children) respiratory samples from 7,139 patients (2,524 adults and 4,615 children), 23 samples from 19 patients (6 adults and 13 children) tested positive in the M. amphoriforme 23S rRNA qPCR.

### TABLE 2: Comparison of M. amphoriforme detection rates for different patient groups tested by culture, 16S rRNA PCR, and qPCR

<table>
<thead>
<tr>
<th>Patient group</th>
<th>% positive samples (no. positive/total no. of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunodeficiency Clinic patients</td>
<td>Culture 11.36 (10/88)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA PCR 19.32 (17/88)</td>
</tr>
<tr>
<td></td>
<td>qPCR 16.09 (14/87)</td>
</tr>
<tr>
<td></td>
<td>Total 19.32 (17/88)</td>
</tr>
<tr>
<td>Chest Clinic patients</td>
<td>NT*</td>
</tr>
<tr>
<td></td>
<td>2.70 (1/37)</td>
</tr>
<tr>
<td>Patients with LRTI</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>3.08 (4/80)</td>
</tr>
<tr>
<td>Patients with suspected viral LRTI</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>0.27 (19/7,139)</td>
</tr>
</tbody>
</table>

* NT, not tested.

Aliquots of 2 μl of template DNA were amplified in a 20-μl reaction mixture with 1X Sso Fast mix (Bio-Rad, United Kingdom) and 200 nM each primer (Table 1). The PCRs were carried out in a RotorGene Q thermocycler (Qiagen, United Kingdom) set to a thermal cycling program of 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, with fluorescence detection at an excitation wavelength of 470 nm and an emission wavelength of 510 nm, and a final melt curve analysis. The specificity of the assay was tested in silico and in vitro by amplification of non-M. amphoriforme DNA. The detection and quantification limits of the assay were established on M. amphoriforme NCTC 11740 DNA. To further confirm the specificity of the assays, the identities of the amplicons were confirmed by sequencing.

The 23S rRNA PCR was used for M. amphoriforme identification in RIE respiratory samples for viral screening. The DNA samples were pooled in groups of 10. The DNA from individual samples from positive pools underwent the same 23S rRNA PCR amplification to determine individual results.

**Nucleotide sequence accession numbers.** The M. amphoriforme 16S rRNA sequence from the single sample that was positive for M. amphoriforme among patients attending the RFL Chest Clinic was deposited with GenBank and assigned the accession number HM235449. Among the patients with suspected LRTI, the M. amphoriforme rRNA sequences for the four positive samples were submitted to GenBank and assigned accession numbers HM235442 to HM235446. The GenBank accession numbers for M. amphoriforme 16S rRNA sequences or the udg gene (n = 14 and 1, respectively) from samples from the RFL Immunodeficiency Clinic patient group are HM235425 to HM235439.

### RESULTS

**Analytical specificity and sensitivity of M. amphoriforme-specific qPCR assays.** We designed two novel qPCR assays for the identification of M. amphoriforme among patients attending the RFL Chest Clinic. PCR products were, however, obtained for 10 patients (37 samples out of 278; 13.3%), 16S rRNA PCR was positive for 17 patients (70/275; 25.5%), and qPCR was positive for 14 patients (64/263; 24.3%). These results are summarized in Table 2. Multiple samples tested positive for 11 of the 13 patients for whom multiple samples were received, with positivity lasting for 197 to 1,627 days. Estimated bacterial loads were ≥10⁵ organisms per ml of sputum in at least one sample for 10 positive patients. Routine microbiology results were available for 70 samples from 15 M. amphoriforme-positive patients and for 39 samples from 18 matched negative patients. Known respiratory pathogens, including H. influenzae, S. pneumoniae, M. catarrhalis, and P. aeruginosa, were found in more M. amphoriforme-negative sputum samples (59%) than in M. amphoriforme-positive samples (24%). H. influenzae was the most commonly isolated pathogen (18% of all samples) and was found significantly less often in M. amphoriforme-positive samples (P = 0.003, Fisher’s exact test).

**M. amphoriforme in patients attending the RFL Chest Clinic.** A total of 38 sputum samples from 37 patients attending the RFL Chest Clinic were tested. Of these patients, 17 had a diagnosis of chronic obstructive pulmonary disease, 14 had bronchiectasis, and 1 patient had both conditions. Culture results indicated normal respiratory tract flora in 15 samples, H. influenzae in 4 samples, 4 samples with S. aureus, and 15 samples with single isolates of Acinetobacter, Pseudomonas, or Citrobacter sp. In this group, there was a single sample positive for M. amphoriforme (Table 2): a patient who had been taking clarithromycin for an exacerbation of symptoms and in whom no other significant pathogen was found.

**M. amphoriforme in patients with suspected LRTI.** M. amphoriforme was detected in 4 (5%) out of 80 patients with LRTI (who had been recruited from general practices): 1/80 throat swabs and 3/50 sputum samples were positive by 16S rRNA PCR (Table 2). The identities of all PCR-positive amplicons were confirmed by sequencing, and the sequences were submitted to GenBank. None of the control samples (49 throat swabs from healthy individuals) were positive. All four M. amphoriforme-positive samples were taken from patients with clinical signs of acute LRTI, including raised pulse rate, respiratory rate, and C-reactive protein concentration compared with the controls. None had a history of recent travel, alcohol consumption, or steroid treatment. Other respiratory organisms were detected in two of the M. amphoriforme-positive patients: coronavirus, human rhinovirus, H. influenzae plus Streptococcus pneumoniae in the patient with M. amphoriforme-positive throat swab, and enterovirus in a patient with M. amphoriforme-positive sputum.

**M. amphoriforme in patients with suspected respiratory viral infection.** The respiratory samples screened for suspected LRTI used in this study were collected between 1 March 2011 and 11 March 2012. Out of 10,747 (3,496 from adults and 7,251 from children) respiratory samples from 7,139 patients (2,524 adults and 4,615 children), 23 samples from 19 patients (6 adults and 13 children) tested positive in the M. amphoriforme 23S rRNA qPCR.
(Table 2). The positive sample sources included nasopharyngeal secretions (6), nose throat swabs (1), throat swabs (8), throat swabs for virology (3), sputum (1), and induced sputum (1), and they originated from the Accident and Emergency Unit (12), Intensive Treatment Unit/High Dependency Unit (4), Children’s Ward (4), Infectious Diseases (1), Hematology (1), and Neonatal Unit (1). No other respiratory pathogen was found in 13 samples, and 10 samples had viral coinfection, as detected by real-time PCR, with the following viruses: rhinovirus (4), RSV (3), adenovirus and influenza B viruses (1), human metapneumovirus (hMPV) (1) and parainfluenza virus 2 (1).

**DISCUSSION**

To better understand the epidemiology and pathogenesis of *M. amorphiforme* infection, it is necessary to develop new sensitive and quantitative tools for diagnosis. Due to the fastidious growth of human mycoplasmas, sensitive molecular tools are an essential prerequisite for their identification in order to diagnose infection in a timely manner, so that antimicrobial treatment can be initiated. In this paper, we have described two real-time PCR assays, defined their specificity, and described our evaluation of them in a clinical practice environment.

**Specificity of the qPCR assays.** The qPCR assays we used target the *udg* gene and the *M. amorphiforme*-specific region of the 23S rRNA gene. Both qPCRs were specific for *M. amorphiforme* as they were negative for all other tested species. In contrast, the 16S rRNA PCR was positive for three mycoplasma-related species: *A. laidlawii*, *M. genitalium*, and *M. alvi*. *A. laidlawii* can be found in the human oropharynx, and although *M. genitalium* is primarily a genitourinary tract pathogen of humans, there have been reports of its detection in respiratory samples (9); they may therefore represent a possibility for false-positive results. *M. alvi* has only been found in cattle, and there is no evidence of its presence in humans (10). The high specificity of the real-time assays was further confirmed by sequencing of products, which showed that all positive samples contained *M. amorphiforme*-specific sequences.

The assays were able to detect an estimated single to several copies per reaction mixture and can be used to measure the bacterial load. The high sensitivities of the qPCRs may be important in defining the pathogenic potential of *M. amorphiforme* in future studies, as has been the case for other organisms, such as *M. genitalium* (11, 12). Moreover, a sensitive detection method will improve detection if suboptimal samples are used, as it is not yet clear what is the primary niche of *M. amorphiforme* in the human host.

**M. amorphiforme** in samples from patients with immunodeficiency. The 16S rRNA PCR and qPCR provided more sensitive detection than culture, identifying *M. amorphiforme* in 17 patients (25.5% positive samples) and 14 patients (24.3% positive samples), respectively, versus 10 culture-positive patients (13.3% positive samples). The qPCR gave an equivocal signal for one sample and was negative for another sample for two patients for whom only a single sample was available for the analysis. However, these samples were positive by the 16S rRNA PCR but negative by culture. These results may have arisen through undetected inhibition or loss of DNA during extraction. There was a single sample from one patient positive by the 16S rRNA PCR that was not available for the qPCR and that was negative by culture. The high incidence of *M. amorphiforme* (19.3%) in spuTA of PAD patients suggests that it may be an important cause of infection in this patient group. Although it is difficult to assign the clinical significance of *M. amorphiforme* in this complex group of patients, our data show that *M. amorphiforme* can chronically infect PAD patients and may contribute to LRTI and the pathogenesis of lung disease. Further research should be conducted to characterize *M. amorphiforme* pathogenicity in this patient group.

**M. amorphiforme** from samples from the Chest Clinic and from patients with LRTI. A single sample from among those of 37 patients attending the Chest Clinic was positive for *M. amorphiforme*. These patients are known to be susceptible to a wide range of pathogens that cause chronic sepsis, and further studies in larger groups of patients are required. The detection rate of *M. amorphiforme* (5%) in patients with acute signs of LRTI who were recruited from general practices was similar to that of other known respiratory pathogens, including *Haemophilus influenzae* (6%), coronaviruses (6%), and parainfluenza viruses (4%) (5). Coinfections were a common feature of this patient group (22.5% of patients and 4% of controls), and therefore, the coinfection of the *M. amorphiforme*-positive sample with other organisms does not exclude its etiological role in the LRTI. It was notable that *M. amorphiforme* was not detected in control subjects, as these samples were exclusively throat swabs. It opens the possibilities that this observation was due to the sample type or that *M. amorphiforme* is a primary respiratory pathogen. However, one throat swab from a patient was *M. amorphiforme* positive in this study, and throat swabs are recommended for the detection of other *Mycoplasma* spp. (13). In addition, other respiratory pathogens, such as *S. pneumoniae* and viral pathogens, were detected when throat swabs were employed at their expected frequency (5).

**M. amorphiforme** in samples from patients with suspected respiratory viral infection. The largest group in this study was a 1-year collection of 10,747 respiratory samples submitted for virological testing from patients with suspected respiratory infection. Infection with *M. amorphiforme* was found to be uncommon within this group, with an incidence of 0.21%.

The low incidence we found was not likely caused by sample pooling, as this approach has previously proved successful in the detection of hMPV in clinical samples (14). The study described here is a pilot study with sampling protocols and DNA extraction methods that are not yet optimized for this organism. Thus, the low detection rate may have been because this is not an optimal sampling method. Additionally, other *Mycoplasma* species have periodicity in their detection rates, for example, *M. pneumoniae* infection increases in prevalence every 4 to 7 years (15, 16). The longest study period reported here was 1 year; thus, longitudinal studies are now required to elucidate the periodicity of *M. amorphiforme* infection. In this study, positive results were mostly found in children (68% of the positive patients), but this may reflect the distribution of the samples submitted for testing. An age cross-sectional study is now required. Viral coinfection was present in 10 *M. amorphiforme*-positive samples, all from children. Interestingly, viral infection was not detected in any of the *M. amorphiforme*-positive samples from adult patients. The results from this preliminary study will provide the basis for a larger study of a wide range of samples from patients presenting with symptoms and signs of LRTI.

The results reported here are important pilot data for the study of *M. amorphiforme* and the first step in understanding its wider pathogenicity. Taken together, these data provide support for *M. amorphiforme* as a primary respiratory pathogen. However, these studies should be repeated by other groups in different countries.
and we are currently working with partners to perform such work. The importance of this paper is that it provides a methodology that will assist other groups in diagnosing *M. amphoriforme* infection, and it is only by increasing the number of patients identified with this organism that we will be able to determine its pathogenic potential with certainty.

**ACKNOWLEDGMENTS**

This work was supported by a Peter Samuel Royal Free Fund grant, the Primary Immunodeficiency Association, the Special Trustees of the Royal Free London NHS Foundation Trust, Hampstead, and the University of St. Andrews Medical School.

**REFERENCES**


April 2014 Volume 52 Number 4 jcm.asm.org 1181