

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

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***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 suppurative

Received 4 November 2013 Returned for modification 27 November 2013

Accepted 27 January 2014

Published ahead of print 29 January 2014

Editor: P. H. Gilligan

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doi:10.1128/JCM.03049-13

lung disease (defined as bronchiectasis, lung abscess, or empyema), tuberculosis, immunodeficiency, or previous study participation (in the previous 3 weeks). Age-, sex-, and season-matched controls were recruited from general practice patients attending the clinic for nonrespiratory and noninfective illnesses as well as other healthy volunteers with no history of respiratory tract symptoms for the 2 months prior to recruitment; these control subjects were recruited using the same exclusion criteria as previously described for patients (5).

Respiratory samples (coded for anonymity) submitted for viral diagnosis, including sputum, nasopharyngeal secretions, and throat swabs collected at hospital and primary care settings in southeast Scotland from adults and children referred to the RIE Specialist Virology Centre (SVC) with suspected respiratory infection, were tested. The ages of the patients ranged from 0 to 96 years, with a mean of 19.91 years. The stored data for these samples include the age group, partial postal code, any recorded symptoms or clinical information, referral source, month of sample collection, and results of other virological testing of the sample.

Control organisms. The following control organisms were used to test the specificity of the assays: *M. amphoriforme* NCTC 11740 and *Mycoplasma pneumoniae* ATCC 5167 (Mycoplasma Experience Ltd., United Kingdom); *Mycoplasma testudinis* NCTC 11701 and *Mycoplasma alvi* ATCC 29626 (Leahurst, United Kingdom); *Acholeplasma laidlawii* ATCC 23206, *Mycoplasma buccale* ATCC 23636, *Mycoplasma faucium* ATCC 25293, *Mycoplasma fermentans* ATCC 19989, *Mycoplasma genitalium* ATCC 33530, *Mycoplasma hominis* ATCC 23114, *Mycoplasma orale* ATCC 23714, *Mycoplasma pirum* ATCC 25960, *Mycoplasma pneumoniae* NCTC 10119, and *Mycoplasma salivarium* ATCC 23064 (Public Health England, United Kingdom); *Streptococcus pneumoniae* ATCC 49619, *Klebsiella* sp. ATCC 700603, *Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* NCTC 10662, *Haemophilus influenzae* NCTC 11931, *Legionella pneumophila* NCTC 11192, *Neisseria gonorrhoeae* NCTC 12700, and *Mycobacterium tuberculosis* H37Rv ATCC 27294 (Department of Microbiology, Royal Free NHS Trust, United Kingdom); coagulase-negative *Staphylococcus*, methicillin-resistant *Staphylococcus aureus*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *Bordetella pertussis*, *Streptococcus pyogenes*, *Acinetobacter* spp., *Corynebacterium* spp., *Proteus mirabilis*, and *Candida albicans* clinical isolates (Department of Microbiology, Royal Free London NHS Foundation Trust); *Pneumocystis jirovecii* clinical isolate (Microbiology Department, Raigmore Hospital, United Kingdom); *Pneumocystis jirovecii*, *Candida* spp., and *Aspergillus fumigatus* (RIE SVC, Edinburgh, United Kingdom); *Chlamydia pneumoniae* SA2f (Clinical Microbiology Department, University College London Hospitals NHS Foundation Trust, United Kingdom). The following viruses were also included for evaluation of assay specificity (all from RIE SVC, Edinburgh, United Kingdom): influenza A virus, influenza B virus, respiratory syncytial virus (RSV), parainfluenza virus (PIV1 to -4), human metapneumovirus, human rhinovirus, human coronavirus (hCoV; 229E, OC43, NL63, HKU1, and hEV), measles virus, mumps virus, and human bocavirus (hBoV, types 1 to 4).

Culture methods. Respiratory samples from patients with PAD were inoculated immediately on Mycoplasma Experience agar (Mycoplasma Experience Ltd., Reigate, United Kingdom) and incubated at 36°C in gas jars containing CO₂ gas packs (Oxoid, Basingstoke, United Kingdom). A small number of cultures that had been stored at 4°C for less than 4 days were included, as this has been shown previously not to affect the viability of *M. amphoriforme* (data not shown). Potential *M. amphoriforme* colonies were detected microscopically at ×40 magnification, and their identities were confirmed by using *M. amphoriforme* 16S rRNA PCR and sequencing. Primary cultures contaminated with other microorganisms were recultured using Sputasol-treated samples that had been stored at -20°C. *Mycoplasma* culture was also performed on 16S rRNA PCR-positive samples from the RFL Chest Clinic, but it was not performed on samples from patients with LRTI in the general practice clinic, as these samples had undergone heat killing prior to storage at -70°C, and it was not performed on RIE respiratory samples submitted for viral diagnosis.

TABLE 1 Oligonucleotides used in this study

Target	Oligonucleotide (5'-3') ^a	Product size (bp)
<i>udg</i>	MAudgF, TGCGGCCGATAAAAACCGAAATAT	92
	MAudgR, TTTTCGAAAAGGGTTTGTACTACCA Probe, FAM-TTGTGCTCATCTTCACCC TTTGTGTGCA-BHQ1	
23S rRNA	Forward, GGGGTTCAAATAACAAGTC	106
	Reverse, CGTGATATATGGCTCTTCG	
16S rRNA	Amph-f, AAGCTAGTAAAGGAAATGTTATT	594
	Amph-r, ACTATAGAAATATAGTC	

^a 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. amphoriforme* 16S rRNA 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. amphoriforme* via the 16S rRNA 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 rRNA gene sequence from the preliminary contiguous *M. amphoriforme* strain A39^T whole-genome sequence obtained from the Wellcome Trust Sanger Institute.

***M. amphoriforme* qPCRs.** The oligonucleotides for the *M. amphoriforme* *udg* quantitative real-time PCR (Table 1) were designed and optimized. The optimized *M. amphoriforme* 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. amphoriforme* 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. amphoriforme* 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 rRNA PCR.

A quantitative real-time PCR assay targeting the variable region of the 23S rRNA gene unique for *M. amphoriforme* was designed and optimized.

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

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

^a NT, not tested.

Aliquots of 2 μ l of template DNA were amplified in a 20- μ l reaction mixture with 1 \times 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](https://www.ncbi.nlm.nih.gov/nuclseq/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](https://www.ncbi.nlm.nih.gov/nuclseq/HM235442) to [HM235446](https://www.ncbi.nlm.nih.gov/nuclseq/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](https://www.ncbi.nlm.nih.gov/nuclseq/HM235425) to [HM235439](https://www.ncbi.nlm.nih.gov/nuclseq/HM235439).

RESULTS

Analytical specificity and sensitivity of *M. amphoriforme*-specific qPCR assays. We designed two novel qPCR assays for the identification of *M. amphoriforme*. Both assays were screened for their specificity *in silico* and experimentally tested against the DNA of 35 isolates, including *Mycoplasma* spp. and respiratory pathogens. Both qPCR assays were positive only for *M. amphoriforme*. PCR products were, however, obtained for *A. laidlawi*, *M. alvi*, and *M. genitalium* with the 16S rRNA PCR. The limit of quantification was 0.01 to 0.1 pg of *M. amphoriforme* DNA, equivalent to 9 to 90 organisms per reaction mixture for the *udg* PCR and 20.6 (95% confidence interval, 2.8 to 149.2) copies per reaction mixture for the 23S rRNA PCR, respectively.

***M. amphoriforme* in patients attending the RFL Immunodeficiency Clinic.** *M. amphoriforme* culture, 16S rRNA PCR, and *udg* qPCR were performed on 281 sputum samples from 88 patients with PAD who attended the RFL Immunodeficiency Clinic. Of these, culture was performed on 278 samples, 16S rRNA PCR was done on 275 samples, and *udg* qPCR was performed on 263 samples. *M. amphoriforme* was detected by culture and/or PCR in the sample from at least 1 of 17 (19.3%) patients. A positive culture was 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 $\geq 10^6$ 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 the *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 swab (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. amphoriforme* 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. amphoriforme*-specific region of the 23S rRNA gene. Both qPCRs were specific for *M. amphoriforme* 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. amphoriforme*-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. amphoriforme* 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. amphoriforme* in the human host.

***M. amphoriforme* in samples from patients with immunodeficiency.** The 16S rRNA PCR and qPCR provided more sensitive detection than culture, identifying *M. amphoriforme* 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. amphoriforme* (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. amphoriforme in this complex group of patients, our data show that *M. amphoriforme* can chronically infect PAD patients and may contribute to LRTI and the pathogenesis of lung disease. Further research should be conducted to characterize *M. amphoriforme* pathogenicity in this patient group.

***M. amphoriforme* 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. amphoriforme*. 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. amphoriforme* (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. amphoriforme*-positive sample with other organisms does not exclude its etiological role in the LRTI. It was notable that *M. amphoriforme* 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. amphoriforme* is a primary respiratory pathogen. However, one throat swab from a patient was *M. amphoriforme* 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. amphoriforme* 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. amphoriforme* 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. amphoriforme* 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. amphoriforme*-positive samples, all from children. Interestingly, viral infection was not detected in any of the *M. amphoriforme*-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. amphoriforme* and the first step in understanding its wider pathogenicity. Taken together, these data provide support for *M. amphoriforme* 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.

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