J Antimicrob Chemother
https://doi.org/10.1093/jac/dkac293

Journal of Antimicrobial Chemotherapy

Phenotypic and genotypic antimicrobial susceptibility patterns of the emerging human respiratory pathogen *Mycoplasma amphoriforme* isolated from the UK and Denmark

Jessica Day[1], Baharak Afshar[1], Richard S. Rowlands[2], Taiba S. Umer[2], Helena Windsor[3], Susanne Paukner[4], Jorgen S. Jensen[5], Owen B. Spiller[1,6], Victoria J. Chalker[1] and Michael L. Beeton[1,2]* on behalf of the ESCMID Study Group for Mycoplasma and Chlamydia Infections (ESGMAC)†

[1]United Kingdom Health Security Agency, Colindale, London, UK; [2]Microbiology and Infection Research Group, Cardiff School of Sport and Health Sciences, Cardiff Metropolitan University, Cardiff, UK; [3]Mycoplasma Experience, Brewer Street Dairy Business Park, Bletchingley, Surrey, UK; [4]Nabriva Therapeutics GmbH, 1110 Vienna, Austria; [5]Research Unit for Reproductive Microbiology, Statens Serum Institut, Copenhagen, DK-2300, Denmark; [6]Cardiff University, Division of Infection and Immunity, Department of Medical Microbiology, University Hospital of Wales, Cardiff, UK

*Corresponding author. E-mail: mbeeton@cardiffmet.ac.uk
†Executive Committee members are listed in the Acknowledgements section.

Received 19 April 2022; accepted 4 August 2022

Objectives: To determine the phenotypic and genotypic antibiotic susceptibility of *Mycoplasma amphoriforme* isolates recovered from patients in the UK and Denmark.

Methods: Seven isolates of *M. amphoriforme* were examined for antimicrobial susceptibility to seven antibiotics using the microbroth dilution assay in line with the CLSI guidelines for mycoplasmas. Each isolate was additionally subjected to WGS to identify resistance-associated mutations. Based on the consensus sequences from the genomic data, PCR primers were designed, and tested, for the amplification of the QRDR within the *parC* gene.

Results: Of the seven isolates investigated, four (57%) were resistant to moxifloxacin (0.5–1 mg/L) and levofloxacin (1–2 mg/L), compared with those that were susceptible (0.03–0.06 and 0.006 mg/L, respectively). Isolate H29 was resistant to five of the seven antibiotics tested: moxifloxacin, 0.5 mg/L; levofloxacin, 2 mg/L; azithromycin, 64 mg/L; erythromycin, 128 mg/L; and clindamycin, 64 mg/L. All isolates were susceptible to tetracycline (0.06 mg/L) and lefamulin (0.001–0.004 mg/L). Mutations from genomic data confirmed the presence of an S89F mutation within the ParC protein among all fluoroquinolone-resistant isolates and an A2059G mutation in the 23S rRNA gene in the macrolide- and lincosamide-resistant isolate H29.

Conclusions: To the best of our knowledge, this is the first time where phenotypic and genotypic resistance data have been paired for *M. amphoriforme* confirming a correlation between the two. These data suggest the need for focused testing and resistance determination of isolates from high-risk patients given the backdrop of a high prevalence of antimicrobial resistance.

Introduction

Mycoplasma amphoriforme was first isolated in 1999 from an immunocompromised patient with chronic bronchitis.[1] Since this initial report, further studies have confirmed the presence of this emerging pathogen within clinical samples by both culture- and molecular-based approaches.[2–7] These studies have shown *M. amphoriforme* predominantly detected within samples from immunocompromised patients or those with pre-existing respiratory complications (20%)[5] and, to a lesser extent, among immunocompetent patients (2%–6%),[2,4] with evidence of transmission among immunocompromised patients attending an outpatients clinic on the same day as well as detections from children from within the same family.[4,8]

Like other mycoplasmas, the lack of a cell wall renders *M. amphoriforme* intrinsically resistant to many antibiotics primarily leaving macrolides, fluoroquinolones and tetracyclines as potential options. Acquired resistance further limits

therapeutic options and has been noted as an area of increasing concern globally with *Mycoplasma pneumoniae* in some geographical locations developing resistance to first-line treatments[9] and *Mycoplasma genitalium* with dual resistance to macrolide and fluoroquinolones being documented.[10] Limited work has been undertaken to look at the susceptibility of *M. amphoriforme*.[4,6,8]

In this study we: (i) determined the MICs of seven antibiotics for seven *M. amphoriforme* isolates from the UK and Denmark; (ii) deduced point mutations associated with resistance; (iii) correlated phenotypic and genotypic resistance; and, finally, (iv) established primers for the amplification of regions associated with fluroquinolone resistance to allow future rapid determination of resistance.

Materials and methods

Bacterial isolates and culture

Seven *M. amphoriforme* isolates were examined in this study, six of which were isolated in the UK. These included the A39 type strain (NCTC 11740),[1,7] A55 and A84.[7] Isolate H04 was isolated from a bronchial alveolar lavage obtained from a 48-year-old male patient admitted on a cardiothoracic ward. Isolate H29 was isolated from a 33-year-old female with common variable immunodeficiency (CVID), recurrent chest infections and receiving a prolonged course of prophylactic azithromycin. No clinical information was available for isolate A70. The final isolate, M5572, was previously isolated in Denmark from the sputum of a patient with bronchitis.[7] *Mycoplasma pneumoniae* M129 (ATCC 29342) was used as a control isolate for antimicrobial susceptibility testing. All isolates were grown statically in Mycoplasma Liquid Medium (MLM) (Mycoplasma Experience, UK) at 37°C under normoxic conditions until signs of a colour change in the medium from orange to yellow indicating growth of viable organisms.

Determination of antimicrobial susceptibility

The MIC values were determined by broth microdilution as per CLSI guidelines for mycoplasmas.[11] MIC values were read at the point when the growth control changed colour, which was approximately 7 days post-inoculation. *Mycoplasma pneumoniae* M129 was used concurrently in assays as a control isolate for antimicrobial susceptibility. Due to the lack of interpretive criteria for MIC values for *M. amphoriforme*, resistance was defined by the presence of a ≥8-fold elevated MIC value relative to baseline MIC ranges.

WGS of M. amphoriforme isolates

Fifty millilitre cultures were grown in MLM until a colour change was noted. Cells were collected by centrifugation at 4800 g for 1 h and resuspended in 400 μL of sterile distilled water for DNA extraction using the Qiagen EZ1 Advanced XL automated extractor utilizing the EZ1 DSP virus kit as per the manufacturer's instructions. Genomic sequencing was undertaken using a Nextera XTv2 library preparation kit with V3 chemistry on an Illumina MiSeq platform. Sequences for *gyrA*, *gyrB*, *parC*, *parE* and the 23S rRNA were aligned for all isolates with Geneious Prime (Version 2022.0.1) and SNPs associated with resistance were determined manually.

Primer design for the amplification of the QRDR of the parC *gene of M. amphoriforme*

To enable future investigators the ability to determine the presence of point mutations associated with fluoroquinolone resistance, a primer

Table 1. MIC values and mechanisms of resistance for *M. amphoriforme* isolates

	MIC (mg/L)							Molecular mechanism of resistance	
	moxifloxacin	levofloxacin	tetracycline	azithromycin	erythromycin	clindamycin	lefamulin	amino acid at position 89 of the ParC protein [GenBank accession number]	nucleotide at position 2059 of the 23S rRNA [GenBank accession number]
M. amphoriforme									
A39 (NCTC 11740)	**0.5**	**2**	0.06	0.03	0.06	0.5	0.001	**phenylalanine (F)** [ON924760]	adenine (A) [ON907762]
A55	**0.5**	**2**	0.06	0.001	0.125	0.5	0.001	**phenylalanine (F)** [ON924764]	adenine (A) [ON937620]
A70	0.03	0.06	0.06	0.001	0.06	0.25	0.001	serine (S) [ON924768]	adenine (A) [ON907802]
A84	**1**	**1**	0.06	0.0005	0.06	0.125	0.001	**phenylalanine (F)** [ON924772]	adenine (A) [ON907815]
H04	0.06	0.06	0.06	0.001	0.125	0.25	0.001	serine (S) [ON924776]	adenine (A) [ON907814]
H29	**0.5**	**2**	0.06	**64**	**128**	**64**	0.004	**phenylalanine (F)** [ON938184]	**guanine (G) [ON907813]**
M5572	0.03	0.06	0.06	0.001	0.125	0.25	0.001	serine (S) [ON924782]	adenine (A) [ON907818]
M. pneumoniae									
M129 (ATCC 29342)	0.06	0.5	0.25	0.001	0.03	0.5	0.001	alanine (A) [NC_000912]	adenine (A) [NC_000912]

Bold type denotes resistance.

Table 2. SNPs within the 23S rRNA gene of seven *M. amphoriforme* isolates

	Nucleotide position																	
Isolate	218	277	650	1231	1570	1602	1607	1610	1869	1885	2046	2059	2185	2201	2224	2721	2855	2869
A39	G	T	A	A	C	C	A	A	C	A	T	A	C	C	G	**C**	C	A
A55	G	T	A	A	C	C	A	A	C	A	T	A	C	C	G	**C**	C	A
A70	G	T	A	A	C	C	A	A	C	A	T	A	C	C	G	T	C	A
A84	G	T	A	A	C	C	A	A	C	A	T	A	C	C	**A**	T	C	A
H04	**A**	T	A	A	C	**T**	A	A	C	A	T	A	C	C	G	T	C	A
H29	G	T	A	A	C	C	A	A	C	A	T	**G**	C	**T**	**A**	T	C	A
M5572	G	**C**	**G**	**G**	**T**	C	**G**	**G**	**T**	**G**	**C**	A	**T**	C	G	T	**T**	**G**

Differences between isolates are indicated in bold. The nucleotide in bold and underlined is predicted to account for the macrolide resistance phenotype in isolate H29 corresponding to 2058 in *E. coli* numbering.

set that amplifies the QRDR of the *parC* gene allowing for sequencing was designed, from genome consensus sequences, and tested. PCRs included GoTaq G2 Green Master Mix (Promega, UK) with a final $MgCl_2$ concentration of 1.5 mM and 0.2 μM for primers MAM_parC-F (5′-AAACCC GTACAACGACGGAT-3′) and MAM_parC-R (5′-TCAGTGTATCGCATGGCAGC-3′). Thermocycling conditions were as follows: 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCRs were then run on a 2% w/v agarose gel and visualized with ethidium bromide and a UV transilluminator. The expected product was 248 bp. To confirm specificity, the PCR products were purified and sequenced as previously described.[2]

Results

MIC data for M. amphoriforme

Only three out of seven isolates (42%) were susceptible to all antibiotics examined (A70, H04 and M5572), with four out of seven isolates (57%) having resistance to one or more antibiotics (Table 1). Three isolates (A39, A55 and A84) had elevated MIC values of the fluroquinolone antibiotics moxifloxacin (range 0.5–1 mg/L) and levofloxacin (1–2 mg/L) and were deemed resistant, compared with the susceptibility of the other isolates (range 0.03–0.06 mg/L for moxifloxacin and 0.06 mg/L for levofloxacin). One isolate, H29, was resistant to moxifloxacin (0.5 mg/L), levofloxacin (2 mg/L), azithromycin (64 mg/L), erythromycin (128 mg/L) and clindamycin (64 mg/L). All isolates were susceptible to tetracycline (0.06 mg/L) and lefamulin (0.001–0.004 mg/L).

Presence of genotypic antibiotic resistance markers among M. amphoriforme sequences

Only the S89F substitution in the QRDR of ParC was consistent between all fluroquinolone-resistant isolates (A39, A55, A84 and H29) (Table 1). Alignment of the 23S rRNA sequence revealed 18 differences between isolates (Table 2). The majority of these (12/18) were specific to the M5572 isolate. Two differences were specific for isolate H04, two were specific for A84 and H29, two were specific for A39 and A55 and two were specific for H29. The SNP at nucleotide A2059G within the H29 23S rRNA sequence corresponded with the macrolide resistance-determining site A2058 of *Escherichia coli*.

Design and testing of parC QRDR PCR primers for fluoroquinolone resistance mutation detection

PCR products for amplification of the QRDR of *parC* yielded a product of approximately 248 bp. The DNA sequence confirmed specificity for the target and amplification of the region of interest when aligned with the data obtained from WGS (data not shown).

Discussion

Several publications have identified the presence of *M. amphoriforme* within patient samples, in the absence of other known pathogens, suggesting a potential role in infectious disease.[1,2,5,6] Although detected on several occasions, there are currently limited data on the antimicrobial susceptibility of this organism. To help guide future treatment of suspected cases, a knowledge of susceptibility patterns and markers for resistance is required.

Resistance to fluoroquinolone antibiotics moxifloxacin and levofloxacin was present in four of the seven isolates. Such fluoroquinolone resistance is reminiscent of the findings presented by Gillespie *et al.*,[8] who identified fluroquinolone resistance mutations among isolates from eight of the nine patients. Macrolide resistance was present in one of our isolates, H29, which was additionally resistant to fluoroquinolone antibiotics and clindamycin. These data were not surprising when considering the immunocompromised CVID patient had been receiving long-term prophylactic azithromycin.

To the best of our knowledge, our study is the first to look at the activity of lefamulin against a panel of *M. amphoriforme* isolates, which is a protein synthesis inhibitor belonging to the pleuromutilin class of antibiotics and approved for the treatment of community-acquired bacterial pneumonia in adults. Our data suggest lefamulin may represent a therapeutic option due to its activity against all antibiotic-resistant isolates in this study, which complements clinical efficacy data in patients with community-acquired bacterial pneumonia and *M. pneumoniae* at baseline.[12,13]

Due to the slow-growing nature of *M. amphoriforme*, phenotypic characterization may not be feasible in many laboratories. For this reason, we examined the presence of mutations associated with resistance to predict resistance. Of the four isolates

with elevated MIC values of fluoroquinolones, an amino acid substitution of serine to a phenylalanine was present at residue 89 (S89F) of the ParC protein. An amino acid substitution at this residue has previously been associated with fluoroquinolone resistance in *M. amphoriforme*,[8] *M. genitalium*[14] and *Ureaplasma* spp.,[15] with varying degrees of impact on MIC values. An A2059G (2058 in *E. coli* numbering) within domain V of the 23S rRNA was identified within isolate H29, which has been reported among other macrolide-resistant mycoplasmas.[16] Our study builds on the work by Gillespie *et al*.,[8] who also used WGS to determine the presence of resistance-associated mutations in the absence of phenotypic data, whereas the study herein includes this important phenotypic data on resistance.

WGS may not be an option in all laboratories; therefore, we designed and confirmed the ability of a primer set to amplify the QRDR of the *parC* gene in which mutations are associated with fluroquinolone resistance in this species. These primers will complement those published by Rehman *et al*.[2] for the amplification of the region surrounding the 2059 residue of the 23S rRNA gene associated with macrolide resistance.

In conclusion, this study correlated MIC values with resistance genotypes and demonstrated that, concordant with other fastidious mycoplasmas, identification of resistance-associated mutations is a feasible and rapid alternative method for inferring antimicrobial resistance. As the role of this organism in human disease becomes more apparent, additional susceptibility data are essential.

Acknowledgements

We would like to thank the ESGMAC Executive Committee members (see below) for approving this work.

ESGMAC Executive Committee members

Michael Beeton, Jorgen Skov Jensen, Tomislav Mestrovic, Sabine Pereyre and Barbara Van Der Pol.

Funding

This project was supported by an internal Research and Innovation Services fund and Development of Impactful Research fund at Cardiff Metropolitan University.

Transparency declarations

S.P. is employed by Nabriva Therapeutics GmbH, which also supplied the lefamulin for these studies. All other authors: none to declare.

References

1 Webster D, Windsor H, Ling C *et al*. Chronic bronchitis in immunocompromised patients: association with a novel *Mycoplasma* species. *Eur J Clin Microbiol Infect Dis* 2003; **22**: 530–4. https://doi.org/10.1007/s10096-003-0980-9

2 Rehman SU, Day J, Afshar B *et al*. Molecular exploration for *Mycoplasma amphoriforme*, *Mycoplasma fermentans* and *Ureaplasma* spp. in patient samples previously investigated for *Mycoplasma pneumoniae* infection. *Clin Microbiol Infect* 2021; **27**: 1697.e1–5. https://doi.org/10.1016/j.cmi.2021.06.021

3 van Schaik ML, Patberg KW, Wallinga JG *et al*. *Mycoplasma amphoriforme* vs *M. pneumoniae*: similarities and differences between patient characteristics in a regional hospital in the Netherlands. *J Med Microbiol* 2018; **67**: 1348–50. https://doi.org/10.1099/jmm.0.000809

4 Katsukawa C, Asai S, Mizutani K *et al*. Novel isolation of *Mycoplasma amphoriforme* from a pediatric patient with protracted bronchitis in Japan. *Jpn J Infect Dis* 2016; **69**: 450–1. https://doi.org/10.7883/yoken.JJID.2016.128

5 Ling CL, Oravcova K, Beattie TF *et al*. Tools for detection of *Mycoplasma amphoriforme*: a primary respiratory pathogen? *J Clin Microbiol* 2014; **52**: 1177–81. https://doi.org/10.1128/JCM.03049-13

6 Pereyre S, Renaudin H, Touati A *et al*. Detection and susceptibility testing of *Mycoplasma amphoriforme* isolates from patients with respiratory tract infections. *Clin Microbiol Infect* 2010; **16**: 1007–9. https://doi.org/10.1111/j.1469-0691.2009.02993.x

7 Pitcher DG, Windsor D, Windsor H *et al*. *Mycoplasma amphoriforme* sp. nov., isolated from a patient with chronic bronchopneumonia. *Int J Syst Evol Microbiol* 2005; **55**: 2589–94. https://doi.org/10.1099/ijs.0.63269-0

8 Gillespie SH, Ling CL, Oravcova K *et al*. Genomic investigations unmask *Mycoplasma amphoriforme*, a new respiratory pathogen. *Clin Infect Dis* 2015; **60**: 381–8. https://doi.org/10.1093/cid/ciu820

9 Rothstein TE, Cunningham SA, Rieke RA *et al*. Macrolide resistance in *Mycoplasma pneumoniae*, Midwestern United States, 2014 to 2021. *Antimicrob Agents Chemother* 2022; **66**: e0243221. https://doi.org/10.1128/aac.02432-21

10 Machalek DA, Tao Y, Shilling H *et al*. Prevalence of mutations associated with resistance to macrolides and fluoroquinolones in *Mycoplasma genitalium*: a systematic review and meta-analysis. *Lancet Infect Dis* 2020; **20**: 1302–14. https://doi.org/10.1016/S1473-3099(20)30154-7

11 CLSI. *Methods for Antimicrobial Susceptibility Testing for Human Mycoplasmas: M43-A*. 2011.

12 Waites KB, Crabb DM, Duffy LB *et al*. *In vitro* activities of lefamulin and other antimicrobial agents against macrolide-susceptible and macrolide-resistant *Mycoplasma pneumoniae* from the United States, Europe, and China. *Antimicrob Agents Chemother* 2017; **61**: 2008–16.

13 Paukner S, Mariano D, Das AF *et al*. Lefamulin in patients with community-acquired bacterial pneumonia caused by atypical respiratory pathogens: pooled results from two phase 3 trials. *Antibiotics (Basel)* 2021; **10**: 1489. https://doi.org/10.3390/antibiotics10121489

14 Hamasuna R, Le PT, Kutsuna S *et al*. Mutations in ParC and GyrA of moxifloxacin-resistant and susceptible *Mycoplasma genitalium* strains. *PLoS One* 2018; **13**: e0198355. https://doi.org/10.1371/journal.pone.0198355

15 Beeton ML, Chalker VJ, Jones LC *et al*. Antibiotic resistance among clinical *Ureaplasma* isolates recovered from neonates in England and Wales between 2007 and 2013. *Antimicrob Agents Chemother* 2016; **60**: 52–6. https://doi.org/10.1128/AAC.00889-15

16 Pereyre S, Tardy F. Integrating the human and animal sides of mycoplasmas resistance to antimicrobials. *Antibiotics (Basel)* 2021; **10**: 1216. https://doi.org/10.3390/antibiotics10101216