Laboratory and Epidemiology Communications

Novel Isolation of *Mycoplasma amphoriforme* from a Pediatric Patient with Protracted Bronchitis in Japan

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Communicated by Makoto Kuroda

Mycoplasma amphoriforme was first isolated in 1999 from an immunocompromised patient with chronic bronchitis in the UK (1). Subsequently, the species was isolated from patients with respiratory tract infections in the UK, Denmark, France, and Tunisia but had not been isolated in Japan (1–3). In this study, we detected *M. amphoriforme* while investigating *Mycoplasma pneumoniae* and reported details regarding both the bacterium and patient.

From October 2013 to January 2016, we investigated 406 patients with suspected *M. pneumoniae* infections, of which 403 patients were aged 15 years or younger and 3 patients were aged 16 years or older, to identify the prevalence of *M. pneumoniae* infections. Throat swabs were collected from 3 hospitals and 6 clinics in Osaka, kept in BD Universal Viral Transport System (Nippon Becton Dickinson Inc., Tokyo, Japan), and cryo-preserved until further examination. DNA was extracted from the specimens using the QIAamp DNA Mini Kit (QIAGEN, Tokyo, Japan) and *M. pneumoniae* genes were detected by real-time quantitative PCR (qPCR) (4). Moreover, the specimens were inoculated in both diphasic mycoplasma medium and PPLO broth (Nippon Becton Dickinson Inc.) that was supplemented with horse serum (Thermo Fisher Scientific K. K., Kanagawa, Japan) and yeast extract, which we prepared using dry yeast (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan) (5). PCR was performed to identify the isolated bacteria (6). Of the 406 specimens, 194 specimens (47.8%) became positive for *M. pneumoniae* using either qPCR or culturing. In detail, 186 specimens were positive for *M. pneumoniae* using qPCR and 188 *M. pneumoniae* isolates were detected by culturing. Moreover, 1 isolate, designated as M151, was obtained by culturing but not identified as *M. pneumoniae* by the

specific PCR. The M151 isolate grew in PPLO broth, but did not grow in the diphasic mycoplasma medium and PPLO agar. Furthermore, the M151 isolate was able to pass through a membrane filter of 0.45- μ m pore size similar to *M. pneumoniae* and catabolized glucose, but could not hydrolyze arginine in the PPLO broth. Therefore, 16S rRNA gene sequence analysis was performed to identify the M151 isolate. The 16S rRNA gene sequence of M151 showed 99.9% identity (1408/1409, partial 16S rRNA gene sequence) to that of the previously reported *M. amphoriforme* A39 (GenBank accession number NR_117836). Furthermore, M151 was qPCR positive for *M. amphoriforme* (7); thus, the isolate M151 was identified as *M. amphoriforme*. The partial 16S rRNA gene sequence of *M. amphoriforme* M151 has been deposited in DDBJ/ENA/GenBank databases under the accession number LC131338.

The minimum inhibitory concentrations (MIC) of erythromycin (EM), clarithromycin (CAM), azithromycin (AZM), clindamycin (CLDM), tetracycline (TC), minocycline (MINO), ciprofloxacin (CPFX), levofloxacin (LVFX), moxifloxacin (MFLX), and gatifloxacin (GFLX) for *M. amphoriforme* M151 were determined by the broth microdilution method using the Dry Plate Eiken (Eiken Chemical Co., Ltd., Tokyo, Japan). The MIC values of the drugs for *M. amphoriforme* M151 were comparable to those for *M. pneumoniae* M129, which was the *M. pneumoniae* susceptible reference strain (Table 1). The isolate M151 was susceptible to antibiotics that are usually used for mycoplasmal infections.

The details of the patient infected with *M. amphoriforme* are explained here. In August 2015, a 4-year-old girl, patient No. 7 (Table 2), visited the Asai Children's Clinic for evaluation of a cough, which had persisted for 3 months without fever, nasal discharge, or other systemic findings. Her white blood cell count was $9.0 \times 10^9/L$ with 53% neutrophils, and her C-reactive protein level was 0 mg/L. She had no abnormal findings in the biochemical tests and had no underlying disease. Her antibody titer for *M. pneumoniae*, assessed using the particle agglutination test (SERODIA MYCO II, Fujirebio Inc., Tokyo, Japan) was 1:320, indicating that she

Accepted June 1, 2016. J-STAGE Advance Publication June 30, 2016.

DOI: 10.7883/yoken.JJID.2016.128

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Table 1. MICs of macrolides, tetracyclines, and fluoroquinolones for *Mycoplasma amphoriforme* M151 compared with those for the *Mycoplasma pneumoniae* M129 susceptible reference strain

Strain	Antibiotic									
	EM	CAM	AZM	CLDM	TC	MINO	CPFX	LVFX	MFLX	GFLX
<i>M. amphoriforme</i> M151	0.008	0.015	≤0.004	0.5	0.5	0.25	0.25	0.12	0.06	0.06
<i>M. pneumoniae</i> M129	0.008	0.008	≤0.004	1	0.5	0.25	1	0.5	0.12	0.06

EM, erythromycin; CAM, clarithromycin; AZM, azithromycin; CLDM, clindamycin; TC, tetracycline; MINO, minocycline; CPFX, ciprofloxacin; LVFX, levofloxacin; MFLX, moxifloxacin; GFLX, gatifloxacin.

Table 2. Nine patients who were positive for *Mycoplasma amphoriforme* by real-time quantitative PCR, which targeted the uracil DNA glycosylase gene

Patient	Age (yr)	Sex	Symptom	<i>M. pneumoniae</i> qPCR	<i>M. pneumoniae</i> culture	<i>M. amphoriforme</i> qPCR
1	3	M	Fever (38.5°C), Cough	—	—	+
2	6	M	Fever (38.0°C), Cough	—	—	+
3	7	M	Fever (39.3°C), Cough, and Lower respiratory tract infection	+	+	+
4	2	F	Fever (40.0°C), Cough, and Pneumonia	—	—	+
5	7	M	Fever (39.3°C), Cough, and Pneumonia	+	+	+
6	4	M	Fever (38.9°C), Cough, and Pneumonia	+	+	+
7	4	F	Cough, Protracted Bronchitis, and No Fever	—	—*	+
8	5	F	Cough, Bronchitis, and No Fever	—	—	+
9	9	F	Cough, Pneumonia, and Fever (unknown)	+	+	+

*: *Mycoplasma amphoriforme* M151 isolated from a throat swab.

was antibody positive for a *M. pneumoniae* infection because results of 1:320 titer or higher with single serum were considered positive. However, she was negative for *M. pneumoniae* by qPCR, which was also not detected by the culture test. A fluoroquinolone antibiotic, tosufloxacin, was administered as an outpatient treatment for 8 days; the symptoms of her cough disappeared and she experienced an uneventful recovery.

Because culturing is difficult for fastidious organisms like *M. amphoriforme* (2), we performed genetic screening for all 406 specimens to detect whether a trace of *M. amphoriforme* was present. We demonstrated that 9 specimens (2.2%) were *M. amphoriforme* DNA positive (Table 2) using qPCR for *M. amphoriforme* (7). However, *M. amphoriforme* was not detected by culturing these specimens, except in the specimen from which *M. amphoriforme* M151 was isolated. Additionally, 4 of these specimens were found to be *M. pneumoniae* positive not only by qPCR for *M. pneumoniae*, but also by culturing (Table 2). Therefore, these 4 patients were suspected to have coinfection with *M. amphoriforme* and *M. pneumoniae*. Patients coinfecting with *M. amphoriforme* and *M. pneumoniae* generally exhibited symptoms of high fever, in contrast to 4 of the 5 patients infected with *M. amphoriforme*, who exhibited no or mild fever. Apart from the *M. amphoriforme* culture-positive patient, the other 8 patients also did not have an underlying disease. Coinfected patient No. 5 was the older brother of patient No. 7, who was *M. amphoriforme* culture positive. Because the antibody titer for *M. pneumoniae* was high in patient No. 7, we considered that there might have been a coinfection with *M. amphoriforme* and *M. pneumoniae* during the 3-month period of protracted bronchitis. The fact that 5 coinfections were identified in this study demonstrated that there is some type of association between these 2 bacteria. The possibility that *M. amphoriforme* exacerbates a *M. pneumoniae* infection is postulated.

Because *M. amphoriforme* is fastidious and catabolizes glucose similar to *M. pneumoniae*, detecting this species from a culture is very difficult. qPCR is an effective tool for detecting *M. amphoriforme* and was used to identify *M. amphoriforme* and *M. pneumoniae* coinfection in this investigation. Viral coinfections have also been reported (7); therefore, further studies on the detection of *M. amphoriforme* in patients with various types of respiratory infections are required.

Acknowledgments We are very grateful to Tsuyoshi Kenri (National Institute of Infectious Diseases, Japan) for his valuable suggestions with regard to the experiments. This study was supported by JSPS KAKENHI Grant Number 25460834, in part by JSPS KAKENHI Grant Number 24590843 and 15K08835, and also supported in part by the grant of Osaka Foundation for the Prevention of Cancer and Cardiovascular Diseases.

Conflict of interest None to declare.

REFERENCES

- 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.
- 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.
- 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.
- Winchell JM, Thurman KA, Mitchell SL, et al. Evaluation of three real-time PCR assays for detection of *Mycoplasma pneumoniae* in an outbreak investigation. *J Clin Microbiol*. 2008;46:3116-8.
- Craven RB, Wenzel RP, Calhoun AM, et al. Comparison of the sensitivity of two methods for isolation of *Mycoplasma pneumoniae*. *J Clin Microbiol*. 1976;4:225-6.
- Tjhi JH, van Kuppeveld FJ, Roosendaal R, et al. Direct PCR enables detection of *Mycoplasma pneumoniae* in patients with respiratory tract infections. *J Clin Microbiol*. 1994;32:11-6.
- 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.