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Clinical Evaluation of a Novel Point-of-Care Assay To Detect *Mycoplasma pneumoniae* and Associated Macrolide-Resistant Mutations

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ABSTRACT The recent increase in macrolide-resistant *Mycoplasma pneumoniae* in Asia has become a continuing problem. A point-of-care testing method that can quickly detect *M. pneumoniae* and macrolide-resistant mutations (MR mutations) is critical for proper antimicrobial use. Smart Gene (Mizuho Medy Co., Ltd., Tosu City, Saga, Japan) is a compact and inexpensive fully automatic gene analyzer that combines amplification with PCR and the quenching probe method to specify the gene and MR mutations simultaneously. We performed a clinical evaluation of this device and its reagents on pediatric patients with suspected *M. pneumoniae* respiratory infections and evaluated the impact of the assay on antimicrobial selection. Using real-time PCR as a comparison control, the sensitivity of Smart Gene was 97.8% (44/45), its specificity was 93.3% (98/105), and its overall concordance rate was 94.7% (142/150). The overall concordance rate of Smart Gene diagnosis of MR mutations in comparison with sequence analysis was 100% (48/48). The ratio of MR mutations was significantly higher at high-level medical institutions than at a primary medical clinic ($P = 0.023$), and changes in antibiotic therapy to drugs other than macrolides were significantly more common in patients with MR mutations ($P = 0.00024$). Smart Gene demonstrated excellent utility in the diagnosis of *M. pneumoniae* and the selection of appropriate antimicrobials for MR mutations at primary medical institutions, which play a central role in community-acquired pneumonia care. The use of this device may reduce referrals to high-level medical institutions for respiratory infections, thereby reducing the medical and economic burdens on patients.

KEYWORDS *Mycoplasma pneumoniae*, genetic point-of-care testing, macrolide resistant, PCR, quenching probe method

Mycoplasma pneumoniae is a pathogen that causes community-acquired pneumonia and bronchitis in children and adolescents (1–4). Since *M. pneumoniae* does not have a cell wall, macrolides are used as first-line therapy (5) instead of β-lactams. However, since around the year 2000, the proportion of patients with macrolide-resistant *M. pneumoniae* (MRMP) has increased, with particular prevalence in Asia (6). MRMP spread nationwide in Japan from 2011 to 2012, raising the macrolide resistance rate to about 90%. The resistance rate gradually decreased thereafter (7) but rose again to 40% in 2019 (8, 9).

Recent guidelines of the Japanese Society for Pediatric Infectious Diseases (JSPID) and the Japanese Society of Mycoplasmology (JSM) recommend either loop-mediated isothermal amplification (LAMP) (10) or quenching probe (QProbe) methods for *M. pneumoniae* diagnosis (11), both of which are nucleic acid amplification methods with high accuracy

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(concordance rates of 98.5% between LAMP and culture and 97.8% between QProbe and culture) (12–14). However, the number of medical institutions that can carry out a nucleic acid amplification method in-house is limited, and many facilities rely on outsourced tests. Direct detection methods that can identify macrolide-resistant mutations (MR mutations) based on the QProbe method have been developed, but the dedicated measuring devices for this task are large and expensive (15, 16). Since *M. pneumoniae* is known to cause community-acquired pneumonia and bronchitis, point-of-care testing (POCT) devices that can detect this pathogen and identify MR mutations at primary medical institutions are needed.

Smart Gene (Mizuho Medy Co., Ltd., Tosu City, Saga, Japan) was developed based on the concept of point-of-care testing for genetic testing, which can automatically perform nucleic acid extraction, amplification, and detection (17). It is a small, lightweight, and inexpensive gene detection device ($5,000 per unit). The reagent used in Smart Gene is also not expensive, at $30 per sample. In contrast, although a reagent for a real-time PCR gene is less expensive (approximately $5 per sample), the real-time PCR gene detection device is quite expensive (approximately $10,000 to $100,000 per unit). The assay combines PCR and the QProbe method, making it possible to simultaneously detect an *M. pneumoniae* gene and MR mutations in approximately 30 to 50 min with a single test. The test time is also considerably shorter than that of real-time PCR. Smart Gene is easy to introduce even at primary medical institutions where genetic testing has been difficult and may be very useful for the treatment of *M. pneumoniae* with properly directed antimicrobials.

This study aimed to evaluate the performance of the Smart Gene assay in the diagnosis of patients suspected of having *M. pneumoniae* infection and assess its impact on antimicrobial selection.

MATERIALS AND METHODS

Ethical approval and consent to participate. The institutional review board of Saga University Hospital approved the present study (approval no. 2018-08-02). This study was registered with the University Hospital Medical Information Network Clinical Trials Registry (no. UMIN000036804 [https://upload.umin.ac.jp/cgi-bin/icdr_e/ctr_view.cgi?recptno=R000041935]) on 20 May 2019. The study methodology was explained to the participants and their parents, and written informed consent was obtained from all participants and their parents or guardians.

Enrollment. From January to December 2019, patients with suspected *M. pneumoniae* infection at 4 pediatric medical institutions (3 primary medical institutions and 1 high-level medical institution) who provided written informed consent were enrolled. Based on dry cough, a fever of 37.5°C or higher, and respiratory symptoms worse than those of the common cold (dyspnea), the attending physicians (pediatricians) made a comprehensive judgment and selected the subjects for examination. The X-ray examinations were performed at the discretion of the attending physicians. The primary medical institutions treated only outpatients, while the high-level medical institution had hospital facilities that were able to accommodate critically ill patients, in addition to treating outpatients. The actual tests were not targeted at inpatients and were performed only in the outpatient setting. Pneumonia severity assessments were classified according to community-acquired pneumonia guidelines set forth by the American Thoracic Society (18). Each attending physician confirmed the subject's prescription and filled out a record sheet for this study.

Fully automatic gene analyzer Smart Gene and MR mutation detection reagent. The measurement operations of Smart Gene were performed according to the manufacturer's instructions. In short, pharyngeal swab samples were collected from patients using a swab (Nipro sponge swab type L; Nipro Co., Ltd., Osaka, Japan) and suspended in 550 μl of an extraction buffer. Four drops (approximately 110 μl) were dropped into the sample spot of the evaluation reagent cartridge. After the sample was absorbed, the cartridge was inserted into Smart Gene, and measurements were performed. All subsequent operations were fully automatic.

The *M. pneumoniae*-specific QProbe binds to the region containing positions 2063 and 2064 of the 23S rRNA gene (position numbers according to GenBank accession no. X68422.1), and its sequence is homologous to that of the wild-type strain. In this study, the PCR conditions were 55°C for 20 s, 95°C for 10 s, 66°C for 20 s, and 95°C for 10 s, repeated 23 times. Finally, *M. pneumoniae* detection was made with an extinction rate at 55°C. If there was *M. pneumoniae* DNA in the sample, the QProbe was quenched at 55°C regardless of the presence or absence of MR mutations. MR mutations were determined as quenching rates at 55°C and 66°C. Without MR mutations, the QProbe quenched at both 55°C and 66°C, but with MR mutations, it quenched only at 55°C. In this way, Smart Gene was able to assess for the presence or absence of both the *M. pneumoniae* gene and MR mutations. The judgment result was displayed on the touchscreen and printed out automatically.

Real-time PCR. DNA was extracted from the residual sample of this evaluation reagent using the QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany). Using 5.0 μl of the extracted DNA as a sample, the next step was performed with real-time PCR targeting the previously reported P1 gene of *M. pneumoniae* (19) on an Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific, MA, USA). For quantification, artificial plasmid DNA containing the amplified region of the reagent was assayed simultaneously in each real-time PCR. The PCR protocol was as follows: following 2 min at 50°C and 10 min at 95°C, 50 cycles were performed at 95°C for 15 s and 60°C for 1 min. The nucleotide sequences used as primers were the F (forward) primer CCA ACC AAA CAA CAA CGT TCA, the R (reverse) primer ACC TTG ACT GGA GGC CGT TA, and the TaqMan probe VIC-TCA ATC CGA ATA ACG GTG ACT TCT TAC CAC TG-TAMRA (6-carboxytetramethylrhodamine).

DNA sequencing. Briefly, an ~0.8-kb portion of the 23S rRNA sequence flanking the nucleotides at the 2063rd and 2064th positions was amplified by nested PCR and subjected to sequence analysis. The first 35-cycle touchdown PCR employed primers Outer-F (GAT AAT ACG ACT CAC TAT AGG GGT GAC ACC TGC CCA GTG C) and Outer-R (GAA ATT AAC CCT CAC TAA AGG GAG CGG TGC AGC TGG CGC TAC AA) (0.2 μM each) with Tks Gflex (TaKaRa Bio Inc., Shiga, Japan) in a 20-μl reaction mixture, using 2 μl of extracted DNA as a template. The second 40-cycle amplification employed primers Inner-F (AAC TAT AAC GGT CCT AAG GTA GCG) and Inner-R (GCT ACA ACT GGA GCA TAA GAG GTG) (0.2 μM each) with PrimeSTAR HS (premix) (TaKaRa Bio Inc.) in a 50-μl reaction mixture, using 1 μl of the first amplification reaction mixture as its template. The second amplification reaction mixture was purified using NucleoSpin gel and PCR cleanup (Macherey-Nagel GmbH, Düren, Germany). The purified product was subjected to bidirectional sequence analysis using the above-mentioned primers Inner-F and Inner-R. The actual sequencing procedure was ordered from Eurofins Genomics KK (Tokyo, Japan). Further details were described previously (20).

Statistical analysis. All analyses were performed using JMP Pro 13 statistical software (SAS Institute Inc., Cary, NC, USA). Hypothesis testing for differences between the two study groups was performed to compare the presence and absence of preceding macrolides. The rate of MR mutations at each medical institution was measured with the chi-square test. The rate of antimicrobial changes other than macrolides was analyzed with the *G* test. The level of statistical significance was set at 0.05 for all tests.

RESULTS

Participant characteristics. The number of patients suspected of having *M. pneumoniae* infection was 150 (81 males and 69 females), all of whom provided informed consent and were enrolled (Table 1). Their median age was 7 years old (interquartile range, 5.0 to 10.0 years old), and two participants over 18 years old were included. Fifty-nine participants had a fever of 37.5°C or higher. All cases had cough symptoms, and only 5 cases had dyspnea. *M. pneumoniae* was detected with Smart Gene in 51 cases (34.0%). Of those positive cases, 26 were marked as having MR mutations. Pneumonia was seen on chest X ray in 87.5% (35/40) of patients in the *M. pneumoniae*-positive group and in 50.0% (22/44) in the *M. pneumoniae*-negative group. According to the severity classification of pneumonia, most of the cases were mild, 2 were severe, and 8 were moderate. Four patients needed to be hospitalized in the general ward. Thirty-one participants had taken macrolides and 12 had taken nonmacrolide antimicrobials at the time of their enrollment in this study.

In the MR mutation group, antimicrobials were newly administered or changed from other drugs in 14 patients with tosufloxacin (TFLX), a new quinolone, and in 10 with minocycline (MINO). As a result, all fevers resolved within 5 days and were finally relieved without any complications.

Detection of the *M. pneumoniae* gene (correlation with real-time PCR). With respect to *M. pneumoniae* diagnosis, the sensitivity of Smart Gene compared with real-time PCR was 97.8% (44/45), its specificity was 93.3% (98/105), and its overall concordance rate was 94.7% (142/150) (Table 2). The genome copy number of one patient who was Smart Gene negative/real-time PCR positive was 7.0×10^1 copies/ml, which was the lowest among the 45 samples that tested positive by real-time PCR. In contrast, *M. pneumoniae* was diagnosed using sequence analysis in 4 of the 7 cases that were Smart Gene positive/real-time PCR negative.

Detection of MR mutations (correlation with sequence). Sequence analysis was performed for 48 cases to evaluate the correlation between Smart Gene and sequence analysis for MR mutations. The overall match rate was 100% (48/48) (Table 3). There were 25 cases with MR mutations, and all of them harbored A2063G mutations.

Macrolide pretreatment and MR mutation rate. The proportion of patients with macrolide pretreatment and MR mutations diagnosed with Smart Gene was 50.0% (13/26),

TABLE 1 Profile of patients with clinically suspected *Mycoplasma pneumoniae* infection[a]

Parameter	Value for Smart Gene result			
	M. pneumoniae positive, MR mutation positive	*M. pneumoniae* positive, MR mutation negative	*M. pneumoniae* negative	Total
No. of eligible patients	26	25	99	150
No. of male/female patients	19/7	13/12	49/50	81/69
Median age (yrs) (IQR)	8.0 (6.0, 10.5)	6.0 (5.0, 8.0)	8.0 (5.0, 10.0)	7.0 (5.0, 10.0)
No. of patients in age group				
1–18 yrs	26	25	97	148
≥18 yrs	0	0	2	2
No. of patients with fever				
<37.5°C	13	8	66	87
≥37.5°C	11	16	32	59
Unknown	2	1	1	4
No. of patients with/without cough	26/0	25/0	99/0	150/0
No. of patients with/without dyspnea	2/24	1/24	2/97	5/145
No. of patients with/without infiltration by chest X ray/with chest X ray not performed	17/3/6	18/2/5	22/22/55	57/27/66
No. of patients with mild/moderate/severe disease	22/4/0	22/3/0	96/1/2	140/8/2
No. of patients with/without hospitalization	2/24	0/25	2/97	4/146
No. of patients with preceding antimicrobial(s)				
MLs	12	2	14	28
MLs + β-lactams	0	0	2	2
MLs + NQs	1	0	0	1
β-Lactams	2	4	4	10
β-Lactams + NQs	1	0	1	2
None	10	18	77	105
Unknown	0	1	1	2

[a]MR, macrolide resistant; IQR, interquartile range; MLs, macrolides; NQs, new quinolones.

compared with 8.0% (2/25) in patients judged not to have MR mutations ($P = 0.023$). Of the 13 patients with macrolide pretreatment and MR mutations diagnosed with Smart Gene, 10 had their regimens changed to nonmacrolides based on their test results. The new therapies were MINO in 7 cases and TFLX in 3 cases. More patients with MR mutations required a change in drug therapy than with no MR mutations or non-*M. pneumoniae* infections ($P = 0.00024$) (Table 4).

Rate of MR mutations at each medical institution. Table 5 shows the ratios of MR mutations to nonmutated strains at each medical institution. The rate of MR mutations was 100% (5/5) at the high-level medical institution and 45.7% (21/46) at the primary medical institutions ($P = 0.023$).

TABLE 2 *Mycoplasma pneumoniae* detection results of Smart Gene and real-time PCR

Real-time PCR result	No. of patients with Smart Gene result		
	M. pneumoniae positive	*M. pneumoniae* negative	Total
M. pneumoniae positive	44	1	45
M. pneumoniae negative	7	98	105
Total	51	99	150

TABLE 3 Macrolide-resistant mutation detection results of Smart Gene and sequencing[a]

	No. of patients with Smart Gene result		
Sequencing result	**MR mutation positive**	**MR mutation negative**	**Total**
MR mutation positive	25	0	25
MR mutation negative	0	23	23
Not detected	1	2	3
Total	26	25	51

[a]MR, macrolide resistant.

DISCUSSION

The present study is significant for two important reasons. First, the small and inexpensive Smart Gene assay was able to adequately detect *M. pneumoniae* infection and MR mutations in the clinical setting. Second, the use of Smart Gene in patients suspected of having *M. pneumoniae* infection may contribute to starting an appropriate antimicrobial regimen, thereby reducing the number of referrals to high-level medical institutions for respiratory infections.

Testing methods for *M. pneumoniae* in Japan include the culture method, serum antibody tests, antigen tests, and nucleic acid detection methods (21). Since it usually takes 1 week to 1 month to perform cultures and generate serum antibodies, the diagnosis of *M. pneumoniae* in patients with acute infections is often delayed. According to the guidelines of the JSPID and JSM, the QProbe method is recommended for *M. pneumoniae* diagnosis (12, 13). Smart Gene can rapidly detect the presence or absence of the *M. pneumoniae* gene. The present study showed highly accurate results in comparison with real-time PCR, with a sensitivity of 97.8% and a specificity of 93.3%.

The JSPID and JSM recommend TFLX or MINO if fever does not resolve within 2 to 3 days of the administration of macrolides as first-line drug therapy (12, 13, 22, 23). However, TFLX and MINO have relatively high MICs (24), and MINO is contraindicated for children under 8 years of age due to its side effects. MINO and TFLX as first-line therapies for *M. pneumoniae* infections should therefore be considered with caution (12, 13). It has been reported that macrolides are more effective than TFLX against *M. pneumoniae* without MR mutations (25–27). Although there is an opinion that macrolides should be used as first-line drugs for MRMP only during febrile periods, we insist on using TFLX or MINO for MRMP. From the viewpoint of proper antimicrobial use, it is very important to be able to easily identify the presence or absence of macrolide resistance when treating *M. pneumoniae* infection. In Japan, MR mutations in MRMP are mainly at positions 2063 and 2064 of 23S rRNA. The A2063G mutation in particular accounts for about 90% of cases (28). The present study showed highly accurate results with an overall match rate of 100% compared with sequencing, and Smart Gene was able to detect A2063G, A2063C, and A2064G. The C2617G mutation is rare in Japan, and it is not very resistant to macrolides; therefore, we have concluded that Smart Gene does not need to detect it.

At the time of the Smart Gene test, the proportion of patients using macrolides was significantly higher in those with MR mutations than in those without. Thirteen patients who

TABLE 4 Antimicrobial changes after the detection of *Mycoplasma pneumoniae* with the Smart Gene assay[a]

Smart Gene result	**% of patients with preceding MLs (no. of patients with preceding MLs/total no. of patients)**	**Antimicrobials after Smart Gene (no. of patients)**
MR mutation positive	50.0 (13/26)	MLs (3), MINO (7), TFLX (3)
MR mutation negative	8.0 (2/25)	MLs (2)
M. pneumoniae negative	16.2 (16/99)	MLs (3), MINO (5), TFLX (6), none (2)

[a]The *P* value for the comparison of MR mutation results was 0.00024 by a *G* test. MR, macrolide resistant; MLs, macrolides; MINO, minocycline; TFLX, tosufloxacin.

TABLE 5 Ratios of macrolide-resistant mutations with Smart Gene at each medical institution[a]

Facility type	No. of patients with Smart Gene result			Prevalence of MR mutations (%) (no. of patients with MR mutations/total no. of patients)
	MR mutation positive	MR mutation negative	*M. pneumoniae* negative	
High-level medical institution	5	0	8	100.0 (5/5)
Primary medical clinic	21	25	91	45.7 (21/46)
Total	26	25	99	51.0 (26/51)

[a]The *P* value for the comparison of facilities was 0.023 by a chi-square test. MR, macrolide resistant.

were found to have MR mutations with Smart Gene had been administered macrolides in advance at primary pediatric clinics not included in the study before referral; five of them were referred to the high-level medical institution. Considering the higher rate of MR mutations at the high-level medical institution, it was presumed that patients with MR mutations were introduced to the high-level medical institution and tested with Smart Gene because their fever and respiratory symptoms did not improve despite the use of macrolides. Based on the data described above, if the presence of MR mutations is known at the time of *M. pneumoniae* diagnosis at a primary pediatric clinic, macrolides can be adequately used, and referrals to high-level medical institutions due to poor symptom improvement may decrease. Ten of the 13 patients with MR mutations and 0 of 25 without MR mutations required a change in the antimicrobial regimen ($P = 0.00024$). Diagnosing *M. pneumoniae* infection and MR mutations at the same time is therefore likely to have a significant influence on antimicrobial selection (29), reducing the medical and economic burdens on both patients and parents.

This study has several limitations. First, the subjects were mostly children, and the results of this study therefore cannot be directly applied to adults. Second, this is a study that targeted a small part of Japan, and it is necessary to take into account ethnic and geological differences during national or global analyses.

In conclusion, Smart Gene was found to be useful for the diagnosis of *M. pneumoniae* infection and the selection of appropriate antimicrobials, thereby potentially reinforcing the central role of primary medical institutions in the management of community-acquired pneumonia. Promptly diagnosing or excluding *M. pneumoniae* infection is important in this patient population. The use of this device may reduce referrals to high-level medical institutions for respiratory infections, thereby reducing the medical and economic burdens on patients.

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