pneumoniae could be selected or that the development of *de novo* macrolide resistance occurs during macrolide treatment (6, 15). Even when 25 patients who had received macrolides before collecting nasopharyngeal swab samples were excluded, statistical significance of regional differences was still observed: 0.0% (0 of 9) in Muroran, 5.6% (1 of 18) in Asahikawa, 38.5% (10 of 26) in Sapporo and 100.0% (17 of 17) in Kushiro (Table 5), suggesting the importance of regional differences in the prevalence of MR *M. pneumoniae*. Unfortunately, since the number of patients infected with MR *M. pneumoniae* in Muroran and Asahikawa was too small and since clinics in Kushiro and hospitals in Muroran did not participate in this study, it was impossible to adjust the confounding by using a statistical model. We therefore presented the crude prevalences for each category and some combinations.

All MR *M. pneumoniae*-positive samples have the A2063G mutation in the 23S rRNA gene. The origin of the A2063G mutation is another problem: Is MR *M. pneumoniae* generated *de novo* or do a limited number of clones spread to various regions? The strains of MR *M. pneumoniae* isolated in Kushiro were found to have at least two origins, indicating that the spread of a single clone of MR *M. pneumoniae* could not account for macrolide resistance of *M. pneumoniae* (Table 8).

Our study has several limitations. The number of patients was too small to clarify the prevalence of MR *M. pneumoniae*. In addition, areas that participated in the study were limited. A surveillance program covering much broader areas is needed.

In conclusion, there is a regional difference in the prevalence of MR *M. pneumoniae* in pediatric patients in Hokkaido. After excluding patients who received macrolides before collecting nasopharyngeal swab samples, statistical significance of regional differences was still observed.

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Conflict of interest

Pfizer Inc. provided grants for this study but was not involved in the design of the study or in enrollment of patients, data collection, analysis and interpretation, or preparation of the manuscript.

Appendix

Nobuhisa Ishiguro, Naoko Koseki, Miki Kaiho, Hideaki Kikuta, Takehiro Togashi, Keisuke Morita, Naoko Nagano, Masanori Nakanishi, Kyosuke Hazama, Toru

Watanabe, Satoshi Sasaki, Tadashi Ariga, Akiko Okamura, Shigeru Yamazaki, Satoru Shida, Naofumi Kajii, Tetsuo Nagashima, Mikio Yoshioka, Yutaka Takahashi, Mutsuko Konno, Akihito Ishizaka, Takeyasu Takebayashi, Mutsuo Shibata, Hideto Furuyama, Hiroyuki Sawada, Yoshihiro Matsuzono, Mari Murashita, Tatsuru Yamanaka, Hiroyuki Naito, Yasushi Akutsu, Hayato Aoyagi, Katsuyuki Tobise, Chie Tobise, and Katsumi Azuma are members of Hokkaido Pediatric Respiratory Infection Study Group.

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Figure legend

Figure 1. The locations of four cities (Sapporo, Asahikawa, Kushiro and Muroran) in Hokkaido are shown.

Table 1. Prevalence of macrolide-resistant *M. pneumoniae* in four cities

City	Medical institutions	Hospital / Clinic	Number of MR/total isolates (%)	Number of MR/total isolates in the city (%)
Sapporo	A	Hospital	3/5 (60.0%)	21/38 (55.3%)
	B	Hospital	1/6 (16.7%)	
	C	Hospital	9/11 (81.8%)	
	D	Hospital	5/6 (83.3%)	
	E	Clinic	2/3 (66.7%)	
	F	Clinic	0/1 (0.0%)	
	G	Clinic	0/1 (0.0%)	
	H	Clinic	0/1 (0.0%)	
	I	Clinic	1/2 (50.0%)	
	J	Clinic	0/2 (0.0%)	
Asahikawa	K	Hospital	1/5 (20.0%)	1/19 (5.3%)
	L	Clinic	0/14 (0.0%)	
Kushiro	M	Hospital	29/29 (100.0%)	29/29 (100.0%)
Muroran	N	Clinic	0/9 (0.0%)	0/9 (0.0%)
(Total)			51/95 (53.7%)	

*Pvalue by Fisher's exact test <0.05,
**Pvalue by Fisher's exact test <0.008

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Table 2. Prevalence of macrolide-resistant *M. pneumoniae* in outpatients and inpatients

	Total	Macrolide-sensitive	Macrolide-resistant	*P*-value*
Outpatients	70	39 (55.7%)	31 (44.3%)	0.0024*
Inpatients	25	5 (20.0%)	20 (80.0%)	

*P-value by Fisher's exact test

Table 3. Prevalence of macrolide-resistant *M. pneumoniae* in patients visiting hospitals and those visiting clinics

	Total	Macrolide-sensitive	Macrolide-resistant	*P*value*
Patients visiting hospitals	62	14 (22.6%)	48 (77.4%)	< 0.0001*
Patients visiting clinics	33	30 (90.9%)	3 (9.1%)	

*Pvalue by Fisher's exact test

Table 4. Prevalence of macrolide-resistant *M. pneumoniae* in patients who were pre-administered macrolides

	Total	Macrolide-sensitive	Macrolide-resistant	*P*-value*
Pre-administration of macrolide	25	2 (8.0%)	23 (92.0%)	< 0.0001*
No pre-administration of macrolide	70	42 (60.0%)	28 (40.0%)	

*P-value by Fisher's exact test

Table 5. Prevalence of macrolide-resistant *M. pneumoniae* in four cities except for samples from patients in whom macrolides were pre-administered

City	Medical institutions	Hospital / Clinic	Number of MR/total isolates (%)	Number of MR/total isolates in the city (%)
Sapporo	A	Hospital	2/4 (50.0%)	10/26 (38.5%)
	B	Hospital	1/5 (20.0%)	
	C	Hospital	4/6 (66.6%)	
	D	Hospital	1/2 (83.3%)	
	E	Clinic	2/3 (66.7%)	
	F	Clinic	0/1 (0.0%)	
	G	Clinic	0/1 (0.0%)	
	H	Clinic	0/1 (0.0%)	
	I	Clinic	0/1 (0.0%)	
	J	Clinic	0/2 (0.0%)	
Asahikawa	K	Hospital	1/5 (20.0%)	1/18 (5.6%)
	L	Clinic	0/13 (0.0%)	
Kushiro	M	Hospital	17/17 (100.0%)	17/17 (100.0%)
Muroran	N	Clinic	0/9 (0.0%)	0/9 (0.0%)
(Total)			28/70 (40.0%)	

*P value by Fisher's exact test <0.05,
**P value by Fisher's exact test <0.008

Table 6. Prevalence of macrolide-resistant *M. pneumoniae* in patients visiting hospitals and those visiting clinics except for patients in whom macrolides were pre-administered

	Total	Macrolide-sensitive	Macrolide-resistant	*P*value*
Patients visiting hospitals	39	13 (33.3%)	26 (66.7%)	< 0.0001*
Patients visiting clinics	31	29 (93.6%)	2 (6.4%)	

*Pvalue by Fisher's exact test

Table 7. Prevalence of macrolide-resistant *M. pneumoniae* in outpatients and inpatients except for patients in whom macrolides were pre-administered

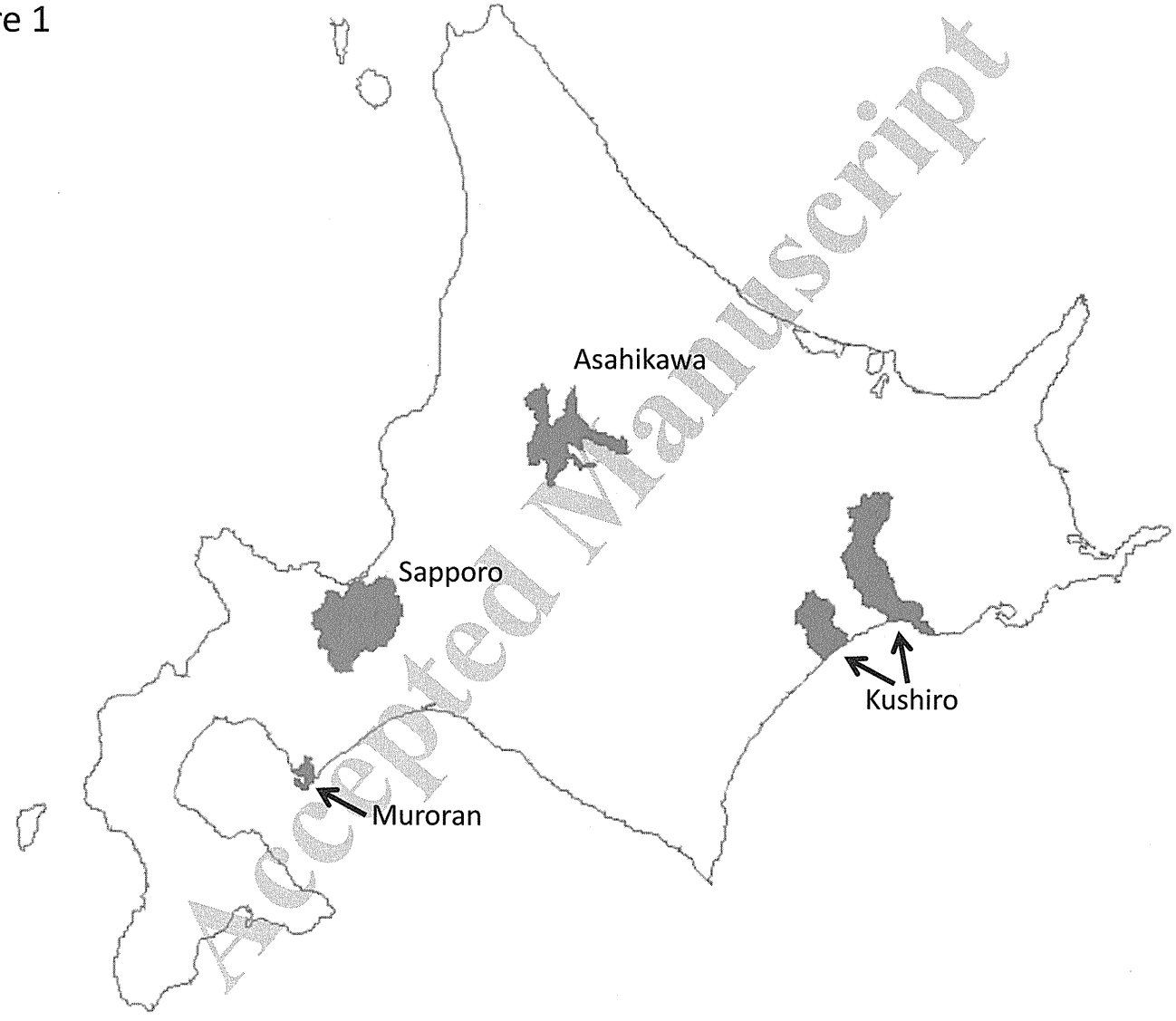
	Total	Macrolide-sensitive	Macrolide-resistant	*P*value*
Outpatients	60	38 (63.3%)	22 (36.7%)	0.1833
Inpatients	10	4 (40.0%)	6 (60.0%)	

*Pvalue by Fisher's exact test

Table 8. *p*1 gene typing of *M. pneumoniae* isolates

Cities	Macrolide sensitive/resistant	Numbers tested	*p*1 gene typing			
			subtype 1	subtype 2	variant 2a	variant 2c
Sapporo	Sensitive	3	1	0	0	2
	Resistant	5	5	0	0	0
Asahikawa	Sensitive	3	3	0	0	0
	Resistant	0	0	0	0	0
Kushiro	Sensitive	0	0	0	0	0
	Resistant	6	4	0	0	2
Muroran	Sensitive	6	0	1	3	2
	Resistant	0	0	0	0	0
Total		23	13	1	3	6

Figure 1



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ORIGINAL ARTICLE

Sensitivity and Specificity of a Loop-Mediated Isothermal Amplification Assay for the Detection of Mycoplasma Pneumonia from Nasopharyngeal Swab Samples Compared with those of Real-time PCR

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SUMMARY

Background: **The aim of this study was to determine the sensitivity and specificity of a loop-mediated isothermal amplification (LAMP) assay kit for the detection of *Mycoplasma pneumonia* (Eiken Chemical Co., Ltd, Tokyo, Japan) from nasopharyngeal swab samples compared with those of real-time PCR.**
Methods: **Nasopharyngeal swab samples taken from 223 patients aged 3 - 18 years who were suspected of having respiratory tract infections associated with *Mycoplasma pneumonia* were used in this study. The samples were tested both by the LAMP assay and by real-time PCR for detection of *Mycoplasma pneumonia*.**
Results: **The sensitivity and specificity of the LAMP assay for the detection of *Mycoplasma pneumonia* were 99.1% (105/106) and 100.0% (117/117), respectively.**
Conclusions: **The LAMP assay for the detection of *Mycoplasma pneumonia* is an accurate and fast assay that is suitable as a diagnostic tool in the acute phase of *Mycoplasma pneumonia* infection.**
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KEY WORDS

loop-mediated isothermal amplification, real-time PCR, detection, *Mycoplasma pneumonia*

INTRODUCTION

Mycoplasma pneumonia is one of the major pathogens of respiratory tract infections, especially in children and young adults [1]. This microorganism is susceptible to macrolides, tetracycline and fluoroquinolones but not to beta-lactam antibiotics [2]. Several methods for diagnosis of *Mycoplasma pneumonia* infections including culture, serological assay, PCR, and real-time PCR are currently available [3,4]. Among these methods, real-time PCR is considered to be the most sensitive method for diagnosis of *Mycoplasma pneumonia* infections [5]. The

Table 1. Quantification of *Mycoplasma pneumonia* DNA and results of the LAMP assay.

Copies/real-time PCR reaction*	Copies/nasopharyngeal sample	Number of samples		LAMP-positive (%)	
10,000 ~	1,500,000 ~	19	79	19 (100.0%)	79 (100.0%)
1,000 ~ 9,999	150,000 ~ 1,499,999	32		32 (100.0%)	
100 ~ 999	15,000 ~ 149,999	28		28 (100.0%)	
1 ~ 99	150 ~ 14,999	27		26 (96.3%)	
Not detected	Not detected	117		0 (0.0%)	

*** Copy numbers were determined by real-time PCR.**

Table 2. Sensitivity and specificity of the LAMP assay for detection of *Mycoplasma pneumonia.*

	Real-time PCR-positive	Real-time PCR-negative	(total)
LAMP positive	105	0	105
LAMP negative	1	117	118
(total)	106	117	223

Sensitivity of the LAMP assay: 99.1% (105/106).
Specificity of the LAMP assay: 100.0% (117/117).
Positive predictive value of the LAMP assay: 100.0% (105/105).
Negative predictive value of the LAMP assay: 99.2% (117/118).

loop-mediated isothermal amplification (LAMP) assay is a novel nucleic acid amplification method developed by Notomi et al. [6]. Recently, a LAMP assay kit for the detection of *Mycoplasma pneumonia* (Eiken Chemical Co., Ltd, Tokyo, Japan) has become commercially available [7]. The aim of this study was to determine the sensitivity and specificity of the LAMP assay kit for the detection of *Mycoplasma pneumonia* from nasopharyngeal swab samples compared with those of real-time PCR.

MATERIALS AND METHODS

Nasopharyngeal swab samples were collected from 223 patients who were suspected of having respiratory tract infections associated with *Mycoplasma pneumonia* from December 1, 2012 to July 31, 2014 at 14 pediatric clinics and in the department of pediatrics in 10 hospitals in Hokkaido, Japan. Each of the nasopharyngeal swab samples was suspended in 3 mL of BD universal viral transport medium (Becton Dickinson, Sparks, MD, USA) before extraction of DNA. DNA was extracted with a QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) from 1 mL of BD universal viral transport medium and was finally resuspended into 50 µL buffer. DNA of *Mycoplasma pneumonia* was identified by using a loop-mediated isothermal amplification (LAMP) assay kit (Eiken Chemical Co., Ltd, Tokyo, Japan) with 5 µL of DNA according to the manual [7]. DNA of *Mycoplasma pneumonia* was also quantified by real-time PCR using Mp181-F and Mp181-R primer pairs and an Mp181-P probe with one µL of DNA as described elsewhere [8]. The sensitivity and specificity of the LAMP assay compared with those of real-time PCR were calculated using JMP software version 11.0.0 (SAS Institute, Cary, NC, USA). Ethical approval for this study was obtained from the Institutional Review Board of Hokkaido University Hospital for Clinical Research.

RESULTS

Mycoplasma pneumonia DNA was detected by real-time PCR from 106 of the 223 samples, and the copy numbers ranged from 6 to 195,584 copies per real-time PCR reaction. The limit of detection of *Mycoplasma pneumonia* DNA by real-time PCR was shown to be one copy per reaction from our preliminary experiment using serial dilutions of a plasmid containing the target sequence. *Mycoplasma pneumonia* DNA was detected by the LAMP assay from all of the 79 samples for which copy numbers were over 100 copies per real-time PCR reaction or 15,000 copies per nasopharyngeal sample and from 26 of the 27 samples for which copy numbers were between 1 and 99 copies per real-time PCR reaction or between 150 and 14,999 copies per nasopharyngeal sample (Table 1). The LAMP assay showed

Table 3. Comparison of real-time PCR and the LAMP assay.

	Real-time PCR	LAMP assay
Number of primers	Two primers from two different regions	Four primers designed to recognize six distinct regions
DNA polymerase	Heat-stable DNA polymerase	DNA polymerase with strand displacement activity
Reaction temperature	Denaturation (95°C), annealing/extension (60°C)	Isothermal reaction at 60 - 65°C
Reaction time	60 - 90 minutes	60 minutes
Method of detection	Fluorescence detection	Turbidimeter
Running cost	$3.6 per reaction	$5.5 per reaction

sensitivity and specificity of 99.1% (105/106) and 100.0% (117/117), respectively, and showed positive and negative predictive values of 100.0% (105/105) and 99.2% (117/118), respectively (Table 2).

DISCUSSION

Mycoplasma pneumonia is one of the common causative agents of respiratory tract infections, especially in pediatric patients. Therefore, rapid and accurate methods for the diagnosis of *Mycoplasma pneumonia* infection are necessary. The LAMP assay can amplify DNA under isothermal conditions with high speed [6] and does not require much laboratory infrastructure. A comparison of real-time PCR and the LAMP assay is shown in Table 3. Several studies have been carried out to evaluate the usefulness of the LAMP assay for the detection of *Mycoplasma pneumonia* compared to the usefulness of conventional PCR using a set of primers targeting the P1 gene [9], nested PCR using two set of primers targeting the P1 and 16S rRNA genes [7], serological assay [10-12], culture [13], and real-time PCR using a set of primers targeting the P1 gene [14,15]. Recently, the LAMP assay kit for the detection of *Mycoplasma pneumonia* (Eiken Chemical Co., Ltd, Tokyo, Japan) using a set of primers targeting the SDC1 repetitive element of the *Mycoplasma pneumonia* genome has become commercially available and is suitable for clinical use from the aspect of quality assurance [7]. The usefulness of this LAMP assay kit has been evaluated in comparison to conventional PCR [9] and serological assay [10-12]. In this study, results obtained by using the LAMP assay kit for the detection of *Mycoplasma pneumonia* (Eiken Chemical Co., Ltd, Tokyo, Japan) were compared with copy numbers of *Mycoplasma pneumonia* DNA measured by real-time PCR using a large number of clinical samples, and we successfully quantified the capacity of the LAMP assay kit for the detection of *Mycoplasma pneumonia*. This study also showed high sensitivity and specificity of the LAMP assay kit for the detection of *Mycoplasma pneumonia*, indicating that the LAMP assay is suitable as a diagnostic tool in the acute phase of *Mycoplasma pneumonia* infection.

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Declaration of Interest:
Loop-mediated isothermal amplification (LAMP) assay kits (Eiken Chemical Co., Ltd, Tokyo, Japan) were provided by Eiken Chemical Co., Ltd.

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Key words
肺炎マイコプラズマ
マクロライド耐性
治療

薬剤耐性の現状と抗菌薬治療

石黒 信久（いしぐろ のぶひさ）*

要旨

道内30余の医療機関に通院・入院した肺炎マイコプラズマ感染症疑い患者724名の患者から鼻咽頭拭い液を採取してマクロライド（ML）耐性率の解析を行うと同時に，各種抗菌薬の治療効果を判定した．肺炎マイコプラズマが検出された検体のうち49.1%はML耐性株（A2063G変異）であったが，ML耐性率には大きな地域差が存在した．治療開始後2日以内に解熱する症例の81%はML感受性株であり，発熱が3日以上持続する症例の83%はML耐性株であった．ML感受性株に感染した症例ではAZM，CAM，MINO，TFLXの効果に有意差はなかった．ML耐性株に感染した症例ではMINOの効果が有意に高かった．

はじめに

2001年にマクロライド（ML）耐性肺炎マイコプラズマが報告されて以降[1]，ML耐性株の増加が大きな問題となっている[2]．ML耐性株は*in vitro*の検査でエリスロマイシン（EM），クラリスロマイシン（CAM），アジスロマイシン（AZM）等のML系抗菌薬に対して耐性を示すが[3]，ML耐性株による呼吸器感染症の治療に対してミノサイクリン（MINO）やトスフロキサシン（TFLX）を必要とするのか等については議論があるところである．小児に対するMINO使用は小児の健康問題（歯牙着色），TFLX使用は関節障害やキノロン耐性菌出現増加の危険性を抱えており，その安易な使用は慎むべきである．これらの理由から，ML耐性肺炎マイコプラズマによる呼吸器感染症の現状把握とその治療方針の作成は急務と考えられる．本稿では，これらの問題に対して自験のデータを元に考察したい．

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I ML耐性肺炎マイコプラズマの現状

1．耐性機序

ML系抗菌薬の作用標的は，細菌の蛋白合成をつかさどるリボソームである．リボソームは16S rRNAと21種の蛋白からなる30Sサブユニットと，5S rRNA，23SrRNAおよび34種の蛋白からなる50Sサブユニットで構成されている（図1）．ML系抗菌薬は23S rRNAのドメインVに結合することによって蛋白合成を阻害する．したがって，ML系抗菌薬がドメインVに結合するうえで重要な塩基に変異が生じると，23S rRNAの立体構造が変化し，その結合率は著しく低下し，耐性が生じる．ML系抗菌薬がドメインVに結合するうえでもっとも重要な塩基は2063番と2064番のアデニンであるとされている（図1）[4]．