〈Others〉

A feasible protocol for identifying macrolide resistant mutations in the 23S rRNA domain V sequence of *Mycoplasma pneumoniae*

Ippei MIYATA and Kazunobu OUCHI

Department of Pediatrics, Kawasaki Medical School

ABSTRACT Continuing a nationwide surveillance of pediatric *Mycoplasma pneumoniae* infections since 2008, our department has been analyzing macrolide-resistance conferring mutations in the pathogen's 23S rRNA gene sequence. There are three target positions, approximately 600 bases apart. We had been reading these positions by amplifying the flanking sequences of these target positions in two different PCR reactions, followed by sequencing of each PCR product, independently. Recent advances have boosted the expected read length of Sanger sequencing using dye-terminators from ~500 bases to ~1 Kb. Owing to a constant demand to process tens of specimens, the authors sought to refine the protocols with an aim to reduce handling time, complexity, and cost. Hereby presented is our refined procedure that enhanced our laboratory's capacity enormously.

doi：10.11482/KMJ-E202147007 *(Accepted on December 21, 2020)*

Key words：*Mycoplasma pneumoniae*, Macrolide resistance, 23S rRNA

INTRODUCTION

Our department has been carrying out a continued nationwide surveillance of pediatric *Mycoplasma pneumoniae* infections since 2008[1－3], collecting samples from our collaborators throughout Japan. This pathogen is known to gain resistance against its first choice antibiotic agent, macrolides, by a single mutation in its 23S rRNA domain V sequence. Currently, three positions, nucleotides A2063, A2064, and C2617 (numbers according to Accession No. X68422.1), are known to be the mutation sites that confer this resistance (Fig. 1A)[4]. To determine whether each of the collected samples harbored *M. pneumoniae*, a real-time PCR detection[5] was carried out. Samples positive for *M. pneumoniae* were further subjected to sequence analysis of the aforementioned three loci. Two independent PCR reactions: one amplifying a portion of the 23S rRNA gene flanking nucleotides 2063 and 2064, and another one amplifying a portion flanking nucleotide 2617 were carried out. The sequences of the two PCR products were then determined by Sanger sequencing.

Sanger sequencing using fluorescent dye-terminators is a common technique for determining DNA sequences. Until the first decade of the 21st

Corresponding author
Ippei Miyata
Department of Pediatrics, Kawasaki Medical School,
577 Matsushima, Kurashiki, 701-0192, Japan
Phone : 81 86 462 1111
Fax : 81 86 464 1038
E-mail: miyata.kkcl@gmail.com

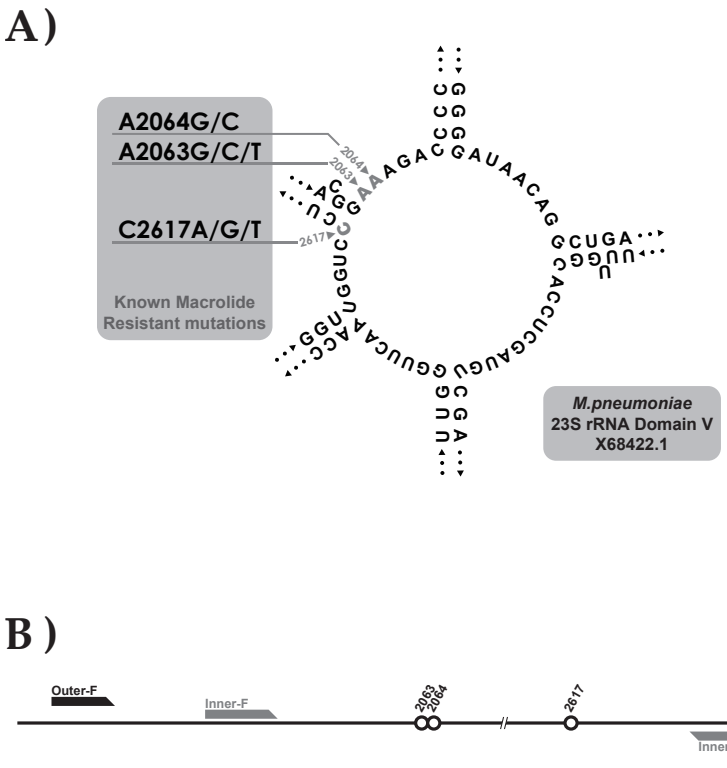

Fig. 1. Macrolide resistant mutation sites and primer positions.
A) Domain V of the 23S rRNA and known macrolide resistant mutations; B) the relative positions of the primers and the mutation sites.

century, the expected read length was ~500 bases per read, which has now advanced to ~1,000 bases per read. The three nucleotide positions of interest in *M. pneumoniae* are approximately 600 bases apart, which necessitated two separate amplifications with different primer pairs and two sequence reads with different primers. However, this protocol was labor intensive when the number of samples was high. Furthermore, there was a risk of confusion regarding PCR products and their corresponding primers for sequencing.

The authors sought to refine, facilitate, and optimize the sequencing procedure as well as increase its scalability for this specific application, taking full advantage of the expected read length of ~1,000 bases per sequence read.

MATERIALS AND METHODS

Template DNA

Crude extracts as well as elaborately purified DNA, are allowed as template DNA. For example, the aforementioned nationwide surveillance collects specimens using BD Universal Viral Transport (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and crude DNA extracted as follows have been successful analytes: 300 μL of the transport medium transferred into a 1.5 mL microcentrifuge tube was spun for 30 min at 4℃, 20,000× *g* in a desktop centrifuge; 285 μL of the supernatant was removed and 85 μL of extraction buffer (Tris-HCl [pH 8.3] 2 mM, KCl 10 mM, $MgCl_2$ 0.045 mM, Triton-X 0.45%, Tween 20 0.45%) supplemented with 1/50th volume of Proteinase K solution (Thermo Fisher Scientific, Inc., Madison, WI, USA; Cat.

Table 1. List of Primers used.

Name	Sequence (5'→3')	Corresponding positions*
Outer-F	GATAATACGACTCACTATAGGGGTGACACCTGCCCAGTGC	1828–1848
Outer-R	GAAATTAACCCTCACTAAAGGGAGCGGTGCAGCTGGCGCTACAA	2722–2701
Inner-F	AACTATAACGGTCCTAAGGTAGCG	1917–1940
Inner-R	GCTACAACTGGAGCATAAGAGGTG	2707–2684

* According to Accession No. X68422.1

Underlined sequences in primers Outer-F and Outer-R denote T7/T3 RNA polymerase promoter sequences, respectively.

No. 25530049) was added to the tubes; followed by thorough mixing by pipetting, the mixture was then transferred to a thin wall 200 μL PCR tube and incubated at 55℃ for 60 min on a heat block; further incubated at 100℃ for 10 min to inactivate Proteinase K; cooled to 4℃and spun down; subsequently analyzed or stored at -80℃.

Nested PCR amplification of sequencing template

All primers used are listed in Table 1 and their positions illustrated in Fig. 1B. The primers were ordered from Eurofin Genomics K. K. (Tokyo, Japan).

Two PCR amplifications were carried out successively. The 1st PCR, (expected product size 895 bp) used primers Outer-F and Outer-R with Tks Gflex (TaKaRa Bio Inc., Shiga, Japan). 2 μL of template DNA was amplified in a 20 μL reaction consisting of 10 μL 2× Buffer, 1 unit Tks Gflex DNA polymerase, and 0.2 μM of each primer. Thermal conditions were as follows: 98℃ for 1 min; 35 cycles of 98℃ for 40 s, 85℃ for 1 s, ramping down at -1℃/s, and 72℃ touching down to 68℃ in 1℃/cycle decrements for 50 s; 68℃ for 1 min. The 2nd PCR, (expected product size 791 bp) used primers Inner-F and Inner-R with high fidelity DNA polymerase, PrimeSTAR HS Premix (TaKaRa Bio Inc.). 1 μL of the 1st PCR product was amplified in a 50 μL reaction consisting of 25 μL PrimeSTAR HS (Premix), and 0.2 μM of each primer. Thermal conditions were as follows: 98℃ for 1 min; 40 cycles of 98℃ for 40 s, 85℃ for 1 s, ramping down at -1℃/s, 55℃ for 5 s, and 72℃ 50 s; 72℃ for 1 min. Both amplifications were carried out on a CFX-96 detection system (Bio-Rad Laboratories, Hercules, CA, USA).

Purification of PCR products for sequencing

The PCR product was purified using Nucleospin Gel and PCR Clean-Up (Machery-Nagel GmbH, Düren, Germany). The first step of the protocol was altered as follows: using a single 200 μL micropipette tip, 100 μL of binding buffer was mixed with the 50 μL 2nd PCR reaction directly in the PCR tube by pipetting up-and-down several times onto the spin column. The subsequent steps followed the manufacturer provided instructions.

Sequencing

Primers "Inner-F" and "Inner-R" were used to sequence the purified PCR product bidirectionally. Sequencing was ordered to Eurofin Genomics K.K., to "buy" time, reliability, and read length.

RESULTS

The nested PCR amplification of sequencing template did not necessarily reduce the bench work considerably since two successive PCR reactions were still required. However, the consumption of extracted DNA was halved.

The modified protocol for PCR product purification produced the following effects. First, by

mixing the PCR reaction and binding buffer in the PCR tube itself, vessels for mixing these fluids were done without, reducing plastic ware consumption/ waste. Furthermore, this resulted in lesser time consumption for bench top setup, and increased free benchtop space, which also boosted the number of processable PCR reactions which could be done in one batch. Second, routine confirmation of purified products by gel electrophoresis was omitted, and substituted by optical density measurement of DNA yield. This reduced the time required for electrophoresis and agarose gel preparation, consumption of agarose and electrophoresis buffer, and lab waste containing DNA staining dyes, which are potential mutagens.

The latest technology enables Sanger sequencing by one primer to encompass all three nucleotides of interest that lie within a 600 base range. Thus, with the same expenditure for one directional sequence read by our previous protocol, a bidirectional read became available, increasing reliability of the results.

DISCUSSION

The two PCR reactions being order dependent, and carried out in different reaction volumes, the risk of misidentifying PCR products was reduced. Furthermore, the inner primers can be used for sequencing of both PCR products, which served as a fail-safe option even when the PCR products of the 1st and 2nd reactions were misidentified. Preliminary experiments (data not shown) proved that first PCR products yielding definite bands on gel electrophoresis could be analyzed without the second PCR, using T7/T3 promoter sequence primers, whereas more among those that did not yield definite bands could be analyzed following the second PCR. Some samples (mostly those proving positive with a low copy number of target DNA by real-time PCR) that could not be analyzed by our previous protocol could now be analyzed by this nested PCR protocol.

The true utility of this specifically optimized protocol is obvious when processing tens of specimens. When identical procedures are frequently repeated, optimizing repeated steps is critical for increasing the overall performance, just as in the case of tuning computer programs for high performance computing[6]. By mixing binding buffer directly in the PCR tube, chances of losing a fraction of the sample that binds to additional plastic ware (the manufacturer provided protocol recommends the mixing be carried out in another microtube) as well as the cost (work load, handling time, additional waste, and monetary expenditure) is reduced. Otherwise, another microtube per specimen is required, which has to be pre-filled with binding buffer; PCR reaction has to be pipetted from the PCR tube, dispensed into the microtube, vortexed, spun down, the mixture pipetted up, and transferred to a spin column; all these steps are abolished. Furthermore, the operator's hands need not put down the micropipette and the PCR tube which results in reduced manipulation time, just like good use of register memory boosts the performance of computer programs. Reduced handling also reduces the chances of contamination. Abolishing the use of microtubes for mixing increased free benchtop area, increasing the number of samples that can be processed in one batch. Costs involved in the washing steps can also be reduced by applying the wash buffer using a single micropipette tip using the "reverse pipetting" technique or by using an automatic dispenser, taking care not to touch the column inner wall. When the number of columns exceeds the capacity of the centrifuge in use, time can further be saved by applying these buffers to waiting columns while others are being centrifuged.

However, as a limitation, this protocol is optimized to suit our laboratory settings; other labs might benefit less. Furthermore, the 2nd PCR reaction might be unnecessary for some samples. Thus, performing

the 2nd PCR reaction for all samples may be a trade-off, depending on settings. When handling samples expected to be of high copy numbers, the 2nd PCR reaction might better be omitted to save time. On the contrary, when the copy numbers are unknown, performing 2nd PCR reaction will minimize the risk of missing analyzable samples. Furthermore, since conditional branching can cause confusion as well as take time for judging, performing 2nd PCR reaction consistently is recommended when handling many specimens.

This protocol has been used successfully for several years in our laboratory causing no trouble and yielding quality results on which a number of publications of our department are based[2, 3, 7, 8]. Even a batch of samples five times the capacity of the centrifuge can be handled by a single operator. A number of laboratory technicians in our department have used this protocol with no difficulty, suggesting its reproducibility, feasibility, robustness, and utility.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare relevant to the content of the manuscript.

REFERENCES

1）Kawai Y, Miyashita N, Kubo M, *et al.*: Nationwide surveillance of macrolide-resistant *Mycoplasma pneumoniae* infection in pediatric patients. Antimicrob Agents Chemother. 2013; 57: 4046-9. doi: 10.1128/AAC.00663-13.

2）Tanaka T, Oishi T, Miyata I, *et al.*: Macrolide-Resistant *Mycoplasma pneumoniae* Infection, Japan, 2008-2015. Emerg Infect Dis. 2017; 23: 1703-1706. doi: 10.3201/eid2310.170106.

3）Nakamura Y, Oishi T, Kaneko K, *et al.*: Recent acute reduction in macrolide-resistant *Mycoplasma pneumoniae* infections among Japanese children. J Infect Chemother. 2021; 27: 271-6, doi: 10.1016/j.jiac.2020.10.007

4）Matsuoka M, Narita M, Okazaki N, *et al.*: Characterization and molecular analysis of macrolide-resistant *Mycoplasma pneumoniae* clinical isolates obtained in Japan. Antimicrob Agents Chemother. 2004; 48: 4624-30. doi: 10.1128/AAC.48.12.4624-4630.2004.

5）Hardegger D, Nadal D, Bossart W, Altwegg M, Dutly F. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. J Microbiol Methods. 2000; 41: 45-51. doi: 10.1016/s0167-7012(00)00135-4.

6）Dowd K: High Performance Computing, First ed, Sebastopol, CA, USA, O'Reilly & Associates Inc. 1993, pp78-79.

7）Oishi T, Takahashi K, Wakabayashi S, *et al.*: Comparing Antimicrobial Susceptibilities among *Mycoplasma pneumoniae* Isolates from Pediatric Patients in Japan between Two Recent Epidemic Periods. Antimicrob Agents Chemother. 2019; 63: e02517-18. doi: 10.1128/AAC.02517-18.

8）Yamaguchi S, Inamitsu T, Inoue S, *et al.*: Pediatric *Mycoplasma pneumoniae* Infection in Primary Care Medical Facilities in Fukuoka. J Ambul Gen Pediatr. 2017; 20:15-21. (Article in Japanese)