Rapid and Accurate detection of Pseudomonas aeruginosa by real-time PCR with Melting Curve Analysis targeting gyrB gene

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Running title: Real-time PCR with MCA for *P. aeruginos*a

Abstract

Laboratory detection of Pseudomonas spp, particularly *P. aeruginosa* is an important assay in the nosocomial control. The study was designed firstly to establish a new assay applied LightCycler PCR technology with melting curve analysis (MCA). A total of 224 gram-negative isolates were used to verify the assay system. The PCR with MCA method using the P. aeruginosa-specific gyrase B gene primers was rapid and accurate; the total run is approximately 3 hours and the sensitivity and specificity relative to the Vitek results were 98.1% and 100%, respectively. Vitek identificaton system was not able to identify the isolates from the new P. otitidis species opposite to the Real-time PCR.

. This assay was validated to be accurate with an overall sensitivity and specificity of 98.7% and 98.9%. Conclusively, this rapid and accurate PCR assay with MCA will help to manage and control infections with *P. aeruginosa.*

Key words: Pseudomonas, melting temperature, taxonomy, gyrase B

Introduction

Strains of *Pseudomonas* (*P.*) *aeruginosa* are one of the most important pathogens, because they are often isolated from various clinical specimens, are resistant to anti-bacterial agents (especially multidrug-resistant), and are common as an important nosocomial pathogen with diverse phenotypes (Pollack 2000). In particular, the bacterium is noted to be the fourth most commonly-isolated nosocomial pathogen, accounting for 10.1% of all hospital-acquired infections according to the CDC. In our hospital, 9.4% of 7,152 bacterial isolates for one year of 2005 were *P. aeruginosa*, implying the third rank following the *Staphylococcus aureus* of 10.3% and the *E. coli* of

10.1%. Since they are usually isolated from compromised patients carrying severe diseases, rapid and precise detection and adequate therapy for the infections are critical for successful outcomes for such patients. All of these issues stress the value of studying how to rapidly and precisely detect *P. aeruginosa*. The current strategy of bacterial identification is now biochemical testing, consisting of primary detection with various culture media and identification using commercial identification systems, such as a Vitek system (bioMerieux, Hazelwood, MO, USA). However, such procedures are not only time- and labor-consuming, but also problematic in diagnostic specificity. Therefore, to overcome these problematic issues, efforts aimed at the species-specific detection of bacterial DNA have been made. For the genetic identification and characterization of bacteria, the 16S rRNA gene is most commonly used, but we pay attention to the *gyrB* gene, because it is rarely transmitted horizontally, its molecular evolution rate is higher than that of 16SrRNA, and the gene is distributed ubiquitously (Anzai et. al, 2000; Kasai et al, 2006). Thus, general bacterial classification is phylogeny based, so that phenetic-based clinical classification of the Pseudomonads has been reported to require consideration of the relationships of the various subbranches (Vandamme et al, 1996). In particular, the laboratory detection of Pseudomonas depends upon the vague and broad definition, subsequently the discrepant results have been reported between standard biochemical and new genetic tests (Qin et. al, 2003; Clarke et al, 2003). Accordingly, to improve the PCR procedures for the genetic detection of *P. aeruginosa* using the *P. aeruginosa gyrB*-specific primers, we applied a LightCycler Technology with high-speed amplification and a melting curve analysis (MCA). Interestingly, the present study happened to present a chance of discovering a novel pseudomonas of P. otitidis being mistaken for P. *aeruginosa*. Accordingly, we here introduce a rapid and accurate genotypic identification procedure by PCR with MCA and discuss the laboratory and clinical relevancy of the genetic assay method.

Materials and Methods

Clinical samples and isolates:

A total of 224 isolates of gram-negative bacteria randomly selected from our stock libraries isolated at the clinical microbiology laboratory, Nagasaki University Hospital from Jan. 2005 to May 2006, were used for the present study. All isolates were identified according to a standard biochemical identification method using standard culture media and a commercial Vitek system (bioMerieux, Hazelwood, Mo, USA) (Kis ka and Gilligan 2003). Briefly, after gram staining, specimens were processed and plated on primary standard media, such as BTB medium (KANTO Chem. Co. Inc, Tokyo, Japan), Chocolate-agar and blood-agar plates (NISSUI Pharmaceutical Co., LTD, Tokyo, Japan). Gram-negative isolated colonies were subcultured onto blood-agar/MacConkey-agar/PASA-agar triplates (Davis et al, 1983) (PASA; P. aeruginosa selective agar including 9-chloro-9-(4-diethlyaminophenyl)-10-phenylacridan. BD, Franklin Lakes, NJ, USA) and were checked for positivity of oxidation and others. Colonies were considered to be *P. aeruginosa* when negative for the gram stain and positive for typical morphology, oxidation, PASA, growth at 42℃, being a lactose nonfermenter, and blue-green color (the 1st step identified isolates). If at least one of the items described above is disagreed, final identification was decided by the test results based on the Vitek system with a gram-negative identification card (the 2nd step identified isolates). Then, 108 clinical sputum specimens freshly obtained from patients with respiratory infections were subjected for direct identification using the new genetic test.

DNA isolation:

Bacterial DNA was extracted from bacteria solution of the 0.5 McFarland concentration using the boiling method (Sulzinski et al, 1997). The DNA extraction from practical clinical specimens was performed using the QIAamp DNA Mini Kit

(QIAGEN, Valencia, CA, USA) according to the manufacturer's instruction. Five µl aliquots of extracted DNA solution were used as the PCR template DNA.

Real-Time PCR/MCA assay:

For amplifying the *P. aeruginosa gyrB* gene, a LightCycler thermal cycle instrument (Roche Applied Science, Mannheim, Germany) equipped with the MCA analysis programme was employed using the primers, cct gac cat ccg tcg cca caa c (gyrB-398) and cgc agc agg atg ccg acg cc (gyrB-620) corresponding to nucleotides 398-620 of the gene (accession No NC_002516) (Qin et al, 2003). Reactions were performed in a 20 µl volume with 0.5 µM primers, 5µl DNA template, 0.25 µM of the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Mannheim, Germany) plus GC Melt (Clontech Laboratories, Mountain View, USA) and DMSO (Wako Co., Lmt.,Osaka, Japan). The reaction conditions were 95℃ for 10 min for activation of the Taq polymerase and then 35 cycles of 10 sec each at 96℃ (denaturation) followed by 12 sec at 72℃ (annealing and extension). After 35 amplification cycles, according to the LightCycler System, the PCR product annealed at 65℃ for 10 seconds was raised from 65℃ to 96℃ at a transition rate of 0.2℃ during continuous fluorescence monitoring at 640 nm after excitation at 483nm. Fluorescence data were analyzed using the LightCycler software (version 3.5, Roche Diagnostics).

To establish a melting temperature (Tm) specific for amplicons, the MCA analysis was conducted ten times using positive and negative controls for *P. aeruginosa*.

Direct DNA sequencing:

To verify the discrepant results between the current biochemical and new genetic analyses, primers were designed based on 16SrRNA gene sequence of P. aeruginosa PAO1 (NC_002516) and they were used to amplify the 16SrDNA of the questionable strains and directly sequenced with the ABI PRISM

Terminator Cycle Sequencing Ready Kit using an Automated DNA Sequence Analyzer (Model 310, Applied Biosystems, Foster, CA, USA).[8] Primers were designed to fractionate the 8 to 1508 region into three parts and each segement was amplified with the following conditions; 94℃ for 2min; 35cycles of 94℃ for 30sec, 58℃ for 30sec and 72℃ for 1.5 min. The primers consisted of 1-S: aga gtt tga tca tgg ctc ag versus 1-AS: ggc tac ctt gtt acg act, 2-R: ttc gta tta ccg cgg atg ct versus 2-F; agc agc cgc ggt aat acg aa, and 3-R; taa ggt tct tcg cgt tgc tt versus 3-F; aac gca acg cga aga acc tta. The sequence homology was compared according to the DDBJ search programme (www.ddbj.nig.ac.jp/search/blast-j.html).

Results

1) Characterization of isolates

A total of 224 isolates were subcategorized according to our criteria; 104 *P.aeruginosa* (1st step identified isolates; 81 cases and 2nd step identified isolates; 23) and 120 non-*P.aeruginosa* gram-negative bacilli. The latter 120 bacilli consisted of 27 non-*P. aeruginosa Pseudomonas* genus (4 *Burkholderia. cepacia*, 3 *Ralstonia. pickettii*, 2 *P. fluorescens*, 13 P. *putida*, 1 *P. stutzeri*, and 3 *Comamonas.testosteroni*, 1 *Sphingomonas. paucimobilis*) 9 *A. baumannii*, 8 *S. maltophilia*, 2 *Achromobacter xylosoxidans*, 1 *Chryseobacterterium indologenes*, 17 *Klebsiella* spp., 8 *Citrobacter* spp., 32 *E. coli*, 4 *M. morganii*, 1 *Proteus mirabilis*, 9 *Enterobacter genus*, and 2 *S. marcescens*.

2) Test and diagnostic validity of PCR with MCA

Using 10 positive controls of the 1st step *P. aeruginosa* isolates and 10 negative control of non-*P. aeruginosa* gram-negative bacillus, the reproducibility and specificity of our PCR-MCA assay were verified. As shown in Fig.1, the positive controls constantly allowed a sharp peak at approximately 88℃ (Fig 1A), but no peaks in the negative controls (Fig 1B). The within-day and between-day reproducibility of the

melting Temperature (Tm) was acceptable with a CV of 0.7% or less. The entire run time was approximately 3.5 hours, 2.5 for the pre-analytical processing of DNA, and 0.5 hours for PCR and MCA analysis.

The lower detection level for *P. aeruginosa* was estimated to be approximately 10^2 bacilli/ml in the dilution manner.

Next, all isolates of 224 were blindly genotyped. The results are summarized in Table 1, implying that the diagnostic sensitivity and specificity relative to standard biochemical culture methods were 98.1% and 100%, respectively. As shown in Table 1, the discrepant outcomes, positive for the Vitek and negative for the genetic test, were derived from 2 isolates (no.11 and 81) out of 23 second step isolates. The 2 isolates showed atypical colony features of morphology and a non-greenish color in the BTB agar plate. The Vitek assay revealed positive data for *P. aeruginosa* with a probability of 93-93.5% according to the Vitek scoring system. However, the system pointed out and warned the negative utilization of D-mannose and positive utilization of ELLM which the findings are unusual for *P. aeruginosa* (Funke G et. al, 2004). The phenetic characteristic features of the 2 isolates are summarized in Table 2.

To confirm which is correct, the 16SrRNA gene was sequenced in the two isolates with discrepant outcomes and one isolate (no.55) with typical findings as a positive control. As shown in Fig.2, the sequence homology for *P. aeruginosa* (NC_002516, Gene ID:3240211) was 100% in the positive control, 98.642%(20 substitution of 1473bps) in the no.11 strain, and 98.713% (19 of 1476 bps) in the no. 81 strain, respectively. On the other hand, the BLAST-SEARCH (DDBJ) hit a novel Pseudomonas species of *Pseudomonas otitidis* (AY-953147) with nucleotide substitution of only 2 bases in the no. 11 strain and with complete accordance in the no. 81 strain.

Then, the diagnostic accuracy of the PCR assay for primary identification was validated using 108 clinical specimens. As shown in Table 3, the culture media grew 258 strains from 108 specimens consisting of 54 *P.aeruginosa* from 54 specimens, 89

other causative bacilli and 65 indigenous bacteria. The PCR assay gave positive signals against P. aeruginosa in 56 specimens of the same 54 specimens as that of the culture and additional 2 specimens. The 2 specimens grew only indigenous bacterium in one specimen and non-P.aeruginosa GNR in the other one.

By using the above mentioned isolates and clinical specimens, the PCR-MCA assay was validated based on the results with the standard culture method. Table 4 shows high accuracy, with an overall sensitivity and specificity of 98.7% and 98.9%.

Discussion

Laboratory detection of *P. aeruginosa* remains an important assay in the patient management and nosocomial infection control. Nowadays, regardless of the vague phenetic definitions for a large number of Pseudomonas spp., identification of bacterial species in hospital microbiology laboratories is mainly based on morphology and biochemical approaches. Moreover, such current methodology remains to be elucidated, especially regarding rapidity, accuracy, objectivity and cost-performance in the detection technology. Recently, the concept of polyphasic taxonomy (Vandamme 1996; Vandamme 2003 which is a classification method based on the data integrating genetic, phenetic and phylogenetic information, is now being introduced into practical microbiology laboratories, implying that genetic testing is indispensable, even at hospital laboratories. Accordingly, we established a new and feasible set of real-time PCR with MCA for the detection of *P. aeruginosa* probably contributing to the resolution of the above problematic aspects. To delineate bacterial species, we used the conserved region of the gyr B gene. Currently, the 16S rRNA, groE, opr and exotoxin genes have been employed as genetic markers for PCR amplification (De Vos et al, 1997; Kurupati et al, 2005; Khan and Cerniglia, 1994). Although the PCR detection methods using such genes have been reported to be sensitive and specific, the gyrB gene coding a type II topoisomerase has recently been noted to be a better candidate for the

identification of bacterial species than 16SrRNA of the standard gene, as described in the Introduction. Indeed, our PCR results, using the gyrB primers, offered an excellent validity for the detection and identification of only *P. aeruginosa* species with the 98% sensitivity and 100% specificity relative to the standard method. The reason why the sensitivity was not 100% was demonstrated to result from the false positive in the Vitek, indicating that the genetic assay is superior. The two isolates discovered, owing to the discrepant results, were confirmed to be a novel Pseudomonas species of *P. otitidis* sp. Nov (Clarke et al, 2006). (accession no AY953147) by 16SrRNA sequencing. In other words, the PCR-MCA assay has been shown to be able to discriminate *P. otitidis* from *P. aeruginosa*. This *P. otitidis,* recently discovered as a causative agent of optic infection, is closely related to, but different from, *P. aeruginosa*. The type strain of *P. otitidis* has been designated as MCC10330^{T}. Our isolates of P. otitidis were also isolated from patients with otitis, and their phenetic characteristics were similar to that of the reports[13]. All of these indicate the necessity of polyphasic examinations, even for routine bacterial identification, in particular for the genus Pseudomonas, because many Pseudomonas species are now being reclassified into separate genera (Vandamme et al, 1996).

Then, the detection sensitivity was 10^2 bacilli/ml, equivalent to the previous reports (Kurupati et al, 2005; Khan and Cerniglia, 1994; Davis et al, 1983). The turn-around-time and labor were also reduced, because the entire run is approximately 3 hours. Considering such characteristics of the assay, we studied whether it is acceptable for the primary detection of P. *aeruginosa* in clinical specimens. Compared to the outcome obtained by the culture test, the PCR test gave a satisfactory diagnostic validity with 100% sensitivity and 96% specificity. Two specimens were discrepant, positive PCR but negative culture. This discrepancy may be related to a property of the PCR assay, in that it can detect dead *P.aeruginosa* or a small number of *P. aeruginosa* that would be under the detection limit of the media test.

Although the genetic test itself is usually estimated to be experimentally significant, it is not practical to apply such a genetic test targeting only one bacillus for all routine specimens. Accordingly, we are now organizing the allotment of roles of culture and genetic tests. That is, the genetic assay is restrictively subjected to emergent specimens and atypical isolates corresponding to the 2nd step of the Vitek.

Finally, this PCR-MCA assay appears promising for the accurate and rapid detection of P. *aeruginosa* in various clinical specimens. At present, an important point is to immediately implement the genetic test under the consideration of scientific and economical aspects.

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Legends

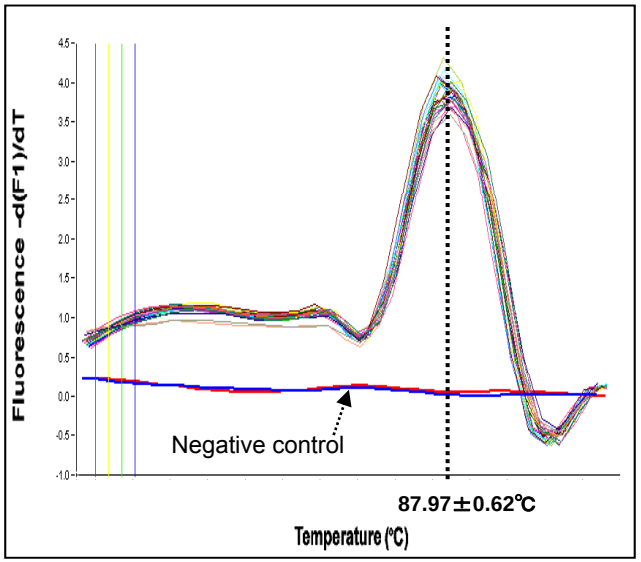
Figure 1: Melting curve analysis (MCA) of gram-negative bacilli,

(A) *P.aeruginosa* positive samples provided the sharp peaks with a constant Tm of 87.9 ℃ and (B) non-P. aeruginosa samples allowed no peak.

Figure 2: Comparison of sequence homology among P.aeruginosa PA01 and the 2 strains of No. 55 and 81.

The BLAST-SEARCH (DDBJ) hit a novel Pseudomonas species of *Peusodomanas otitidis* (AY-953147) with nucleotide substitution of only 2 bases in the no. 11 strain and with complete accordance in the no. 81 strain.

(A) *P. aeruginosa*



(B) gram-negative bacilli except for *P. aeruginosa*

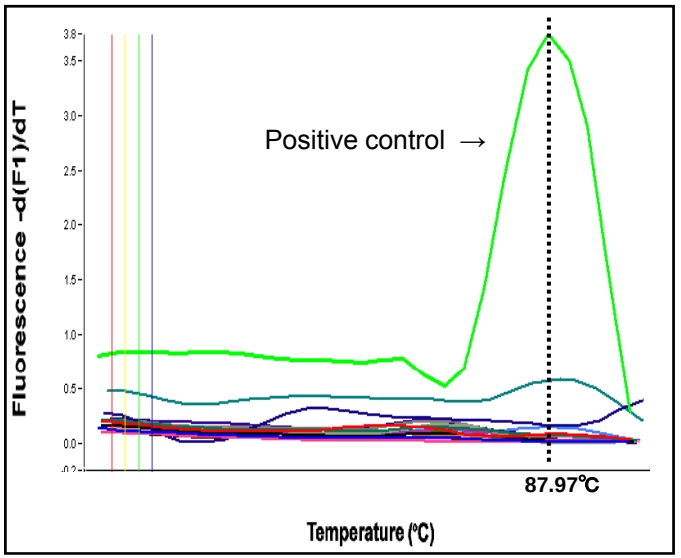


Fig. 1.Motoshima et al.

Table 1. Comparison of the outcomes between the standard biochemical culture method and PCR-MCA assay for 224 isolates of gram-negative rods.

Bacterial strain		**Number of strains**	**gyrB-PCR (%)**	
Pseudomonas aeruginosa		**104**	102	(98.1%)
1st step identification		81	81	(100%)
2nd step identification		23	21	(91.3%)
VITEK2 identification %	≧99%	48	48	(100%)
	＜99% ≧96%	48	48	(100%)
	＜96% ≧93%	8	6	(75%)
Genus Pseudomonas		**27**	0	(0%)
Burkholderia cepacia		4	0	(0%)
Ralstonia pickettii		3	0	(0%)
Pseudomonas fluorescens		2	0	(0%)
Pseudomonas putida		13	0	(0%)
Pseudomonas stutzeri		1	0	(0%)
Comamonas testosteroni		3	0	(0%)
Sphingomonas paucimobili		1	0	(0%)
Other gram-negative bacteria		**93**	0	(0%)
total		**224**		

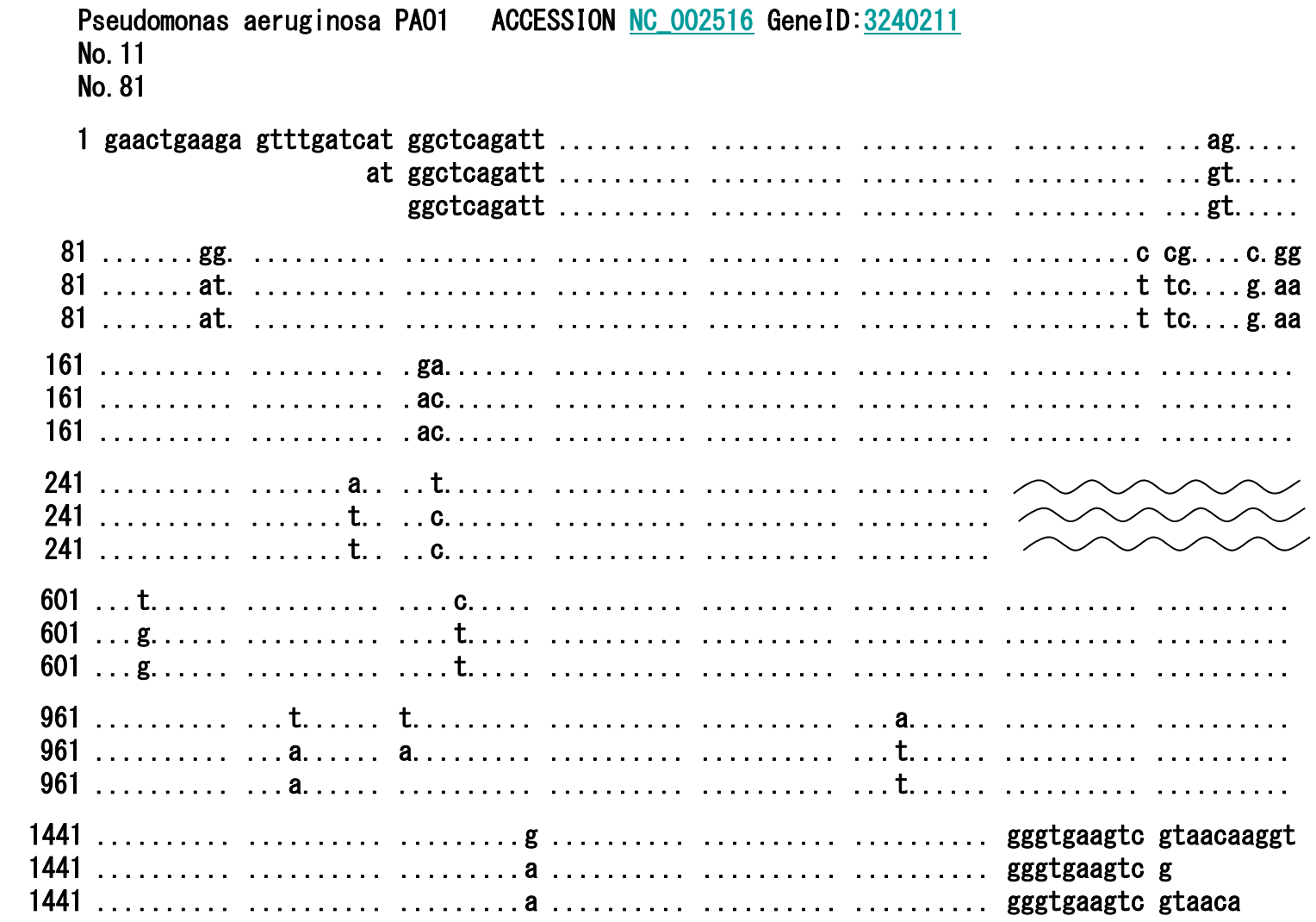


Fig. 2 Motoshima et al.

Table 2. Characterization of the two strains of P. otitidis isolated in the present study

items	P. aeruginosa	P. otitidis	
		no. 11	no.81
Gram(-) rod	yes	yes	yes
Growth			
on MacConkey	yes	yes	yes
on PASA	yes	no	no
at 42℃	yes	yes	yes
Oxidation(oxidase)	yes	yes	yes
Pyocyanin	yes	no	no
Utilization of			
α-D-Lactose	yes	no	no
D-mannose	yes	no	no
ELLM*	no	yes	yes

*;nitrobenzoic acid

Table 3: Comparison of isolation profiles by the culture and PCR assays.
The culture media grew 258 strains from 108 specimens consisting of 54 P.aeruginosa, 89 others, and 65 Indigenous bacteria, while the PCR reacted the same 54 specimens as that of the culture and additional 2 specimens.

main isolated bacteria from 108 clinical samples	culture	PCR-MCA
Pseudomonas aeruginosa	54	54
Pseudomonas fluorescens	1	0
Burkholderia cepacia	1	0
Other gram-negative bacteria.	33	1
Haemophilus influenzae	3	0
Moraxella catarrhalis	2	0
Staphylococcus aureus	23	0
Enterococcus faecalis	6	0
Streptococcus agalactiae	1	0
Streptococcus pneumoniae	2	0
Genus Candida	17	0
indigenous bacteria*)	65	1

*)Nonpathogenic Streptococcus, Neisseria, Haemophilus, CNS(coagulase-negative staphylococcus)and GPR(Gram positive rod) were included in resident microbiota.

Table 4: Summary of a diagnostic validity of the PCR-MCA relative to the results with the standard culture method by using 224 isolates and 108 clinical specimens

subjects	No	positive for *P.aeruginosa*		sensitivity	specificity
		culture	PCR		
Isolates	224	104	102	98.1 %	100%
Specimens	108	54	56	100 %	96.4%
Overall	332	158	158	98.7%	98.9%