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Study on Distribution of Four *Pseudomonas* Species in Living Environment Using Multiplex PCR

Sachiyo Hayashi[1*], Koji Umezawa[1], Osamu Tsuzukibashi[2], Akira Fukatsu[2], Mana Fuchigami[2], Chiaki Komine[2], Hiroshi Yamamoto[3], Mio Hagiwara-Hamano[3], Yukiko Iizuka[3], Satoshi Uchibori[4], Masanobu Wakami[4], Hiroshi Murakami[5], Taira Kobayashi[4], Masahiko Fukumoto[2], Takato Nomoto[1]

[1]Department of Special Needs Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[2]Department of Laboratory Medicine for Dentistry for the Compromised Patient, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[3]Department of Oral Surgery, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[4]Department of Fixed Prosthodontics, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[5]Department of Oral Implantology, Nihon University School of Dentistry at Matsudo, Chiba, Japan
Email: *hayashi.sachiyo@nihon-u.ac.jp

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Abstract

Purpose: The genus *Pseudomonas* is a ubiquitous microorganism frequently detected from immunocompromised patients. The inherent resistance to numerous antimicrobial agents contributes to the opportunistic character of this pathogen exhaustive monitoring of this pathogen is considered of critical importance to public health organizations. The reliable identification method able to distinguish genetic close *Pseudomonas* species is needed, because these organisms are difficult to differentiate by phenotypic or biochemical methods. The purpose of the present study was to design species-specific primers in order to identify and detect four *Pseudomonas* species which are frequently detected from the human oral cavities, and to investigate the distribution of these organisms in the living environment using a multiplex PCR. **Methods:** Polymerase chain reaction (PCR) primers were designed based on partial sequences of the *rpoD* gene of four *Pseudomonas* species. Swab samples were collected from fifty washstands, and the distribution of *Pseudomonas* species was investigated using a conventional PCR at genus level and a multiplex PCR at species level. **Results:** Multiplex PCR method developed in this study was able to distinguish four *Pseudomonas* species clearly. The genus *Pseudomonas* was detected from all samples (100%), whereas *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens* were detected at 44%, 8%, 4% and 2% in fifty swab samples, respectively. **Conclusion:** Our

developed one-step multiplex PCR method is accurate, specific, cost-effective, time-saving, and works without requiring DNA extraction. It was indicated that washstands were the uninhabitable environment for *P. putida*, *P*, *aeruginosa*, *P. stutzeri* and *P. fluorescens*.

Keywords

Genus *Pseudomonas*, Multiplex PCR, Living Environment

1. Introduction

At present, the genus *Pseudomonas* comprises 604 species (https://lpsn.dsmz.de/genus/pseudomonas). This organism can be ubiquitously found in humans, animals, soil, and plants [1] [2]. *Pseudomonas* species were extensively studied for their beneficial or deleterious associations with plants but also for their roles in soil bioremediation due to specific biodegradation properties [3] [4] [5]. Among the genus *Pseudomonas*, some species are detected from humans and animals, and *Pseudomonas aeruginosa* is the most frequently reported pathogen.

The genus *Pseudomonas* is a ubiquitous microorganism frequently detected from immunocompromised patients. The inherent resistance to numerous antimicrobial agents contributes to the opportunistic character of this pathogen. The emergence of healthcare-associated infection is considered a challenge to public health [6]. The problem increases when AmpC-producing *Pseudomonas* species are isolated from the healthcare professionals. The enzyme confers resistance to cephalosporin and penicillin. The bla_{AmpC} genes are found in the chromosome of the genera *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Serratia* and *Providencia*. Bacteria can eventually present sensitivity to cefoxitin, whereas mutant strains express resistance to it due to the loss of repressor genes [7] [8]. Asymptomatic carriers play a fundamental role in the epidemiology of healthcare-associated infection, since they act as disseminators through droplets of saliva expelled during hospital assistance. The asymptomatic colonization may progress to lower respiratory tract infection (pneumonia) and result in health problems to workers and users attended. It is important to establish control strategies to prevent the spread of nosocomial infections.

The detection of the genus *Pseudomonas* at the species level in clinical specimens is important, as it may affect the prognosis and patient management, but identification by conventional biochemical methods can be difficult. The accurate identification and enumeration of *Pseudomonas* species are required to determine their role in various systemic diseases. These organisms can be identified by the sequence analysis of several target genes and MALDI-TOF MS. However, because these methods are laborious, expensive, and time-consuming for use in the detection or discrimination of the clinical isolates of *Pseudomonas*

species at the species level, epidemiological studies on the relationship between these organisms and various diseases are limited. Thus, a simple and more reliable assay for identifying *Pseudomonas* species is required.

Pseudomonas species, especially *Pseudomonas putida*, *P. aeruginosa*, *Pseudomonas stutzeri* and *Pseudomonas fluorescens* are frequently detected from the human oral cavities [9] [10]; however, it remains unclear whether those are an oral resident microorganism or not, and where the source of infection is. The purpose of the present study was to design species-specific primers in order to identify and detect four *Pseudomonas* species, *i.e.*, *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens*, and to investigate the distribution of these organisms in the living environment using a multiplex PCR.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Bacterial strains were obtained from Japan Collection of Microorganisms (JCM; Japan). The following bacterial strains were used in the present study: *P. putida* JCM 13063 and JCM 6156, *P. aeruginosa* JCM 5962 and JCM 2776, *P. fluorescens* JCM 5963 and JCM 13057, and *P. stutzeri* JCM 5965 and JCM 20778. These strains were maintained by cultivating them on Bact™ Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 30˚C overnight under an aerobic condition.

2.2. Design of Species-Specific Primers for Four Pseudomonas Species

Design of species-specific primers for four *Pseudomonas* species, *i.e.*, *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens* was performed as described previously [11]. Briefly, the RNA polymerase sigma factor (*rpoD*) gene sequences of *P. putida* (accession no. AB 039581), *P. aeruginosa* (AB 719996), *P. stutzeri* (KR 780035) and *P. fluorescens* (D 86033) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; *i.e.*, the *rpoD* gene sequences of four *Pseudomonas* species were aligned and analyzed, respectively. Homology among the primers selected for each *Pseudomonas* species and their respective *rpoD* gene sequences was confirmed by a BLAST search.

2.3. Development of a Multiplex PCR Method Using Designed Primers

Bacterial cells were cultured in BHI supplemented with 0.5% yeast extract for 24 h, and 1 ml of the samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 10^7 colony-forming units (CFU)/ml) in 1 ml of sterile distilled water. A total of 3.6 µl of the suspension was then used as a PCR template. The detection limit of PCR was

assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 μM of each primer, 10 μl of 2× MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 5.6 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1× Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker. All experiments were performed in triplicate.

2.4. PCR Analysis for Genus Level Detection of *Pseudomonas* Species

A PCR method for detecting *Pseudomonas* species at the genus level was performed using the previous reported primers [12]. The multiplex PCR mixture contained 0.5 μM of each primer, 10 μl of 2× MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 5.6 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min.

2.5. Environmental Samples

Fifty living environmental samples were collected by wiping the basin and drain of a washstand with a sterile cotton swab, and the distribution of *Pseudomonas* species was investigated using a conventional PCR at genus level and a multiplex PCR at species level. All samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY, USA) and were used as the PCR templates.

3. Results

3.1. Primer Design

Ten specific primers covering the upstream regions of the 1 *rpoD* gene sequences of four *Pseudomonas* species were designed in the present study (**Figure 1**). The specific forward primers were designated as PPF for *P. putida*, PAF for *P. aeruginosa*, PSF for *P. stutzeri* and PFF for *A. P. fluorescens*, whereas the specific reverse primers were designated as PPR for *P. putida*, PAR for *P. aeruginosa*, PSR for *P. stutzeri* and PFR for *A. P. fluorescens*. The amplicon sizes of *P. putida*, *P. stutzeri*, *P. fluorescens* and *P. aeruginosa* were 160 bp, 270 bp, 403 bp and 518 bp, respectively.

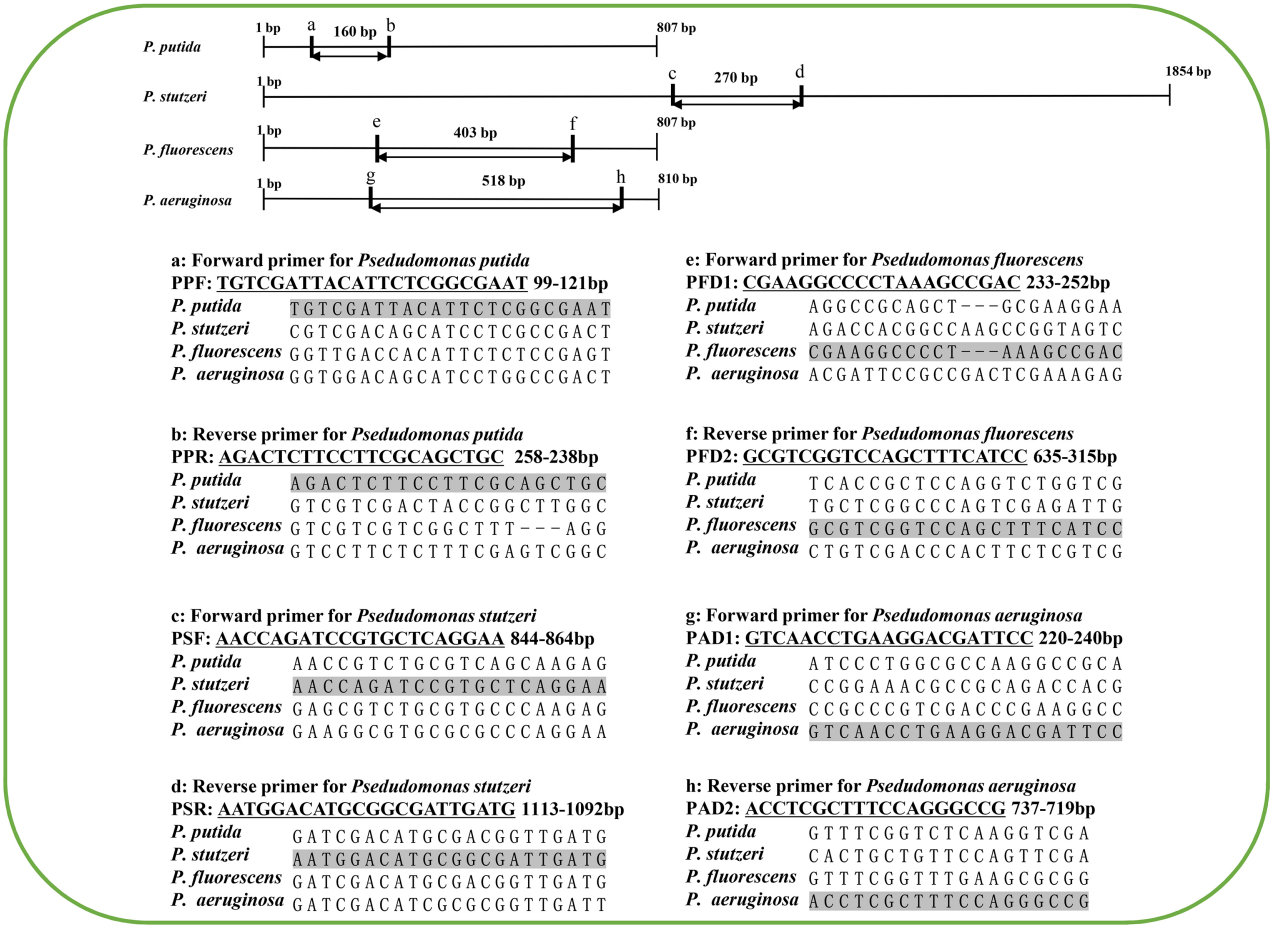

Figure 1. Species-specific primers for *rpoD* gene of four *Pseudomonas* species. The nucleotide sequence of each primer has been underlined.

3.2. Multiplex PCR

Our multiplex PCR method for identifying and detecting four *Pseudomonas* species, *i.e.*, *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens* successfully amplified DNA fragments of the expected size for each species (**Figure 2**). The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 μl) for the *P. putida*-specific primer set with strain JCM 6152, the *P. aeruginosa*-specific primer set with strain JCM 5962, the *P. stutzeri*-specific primer set with strain JCM 5965 and the *P. fluorescens*-specific primer set with strain JCM 5963 (data not shown).

3.3. Distribution of Four *Pseudomonas* Species in Living Environment

Table 1 shows the detection frequency of four *Pseudomonas* species in fifty washstand samples. The genus *Pseudomonas* was detected from all samples. *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens* were detected at 44.8%, 8.0%, 4.0% and 2.0% in fifty swab samples, respectively.

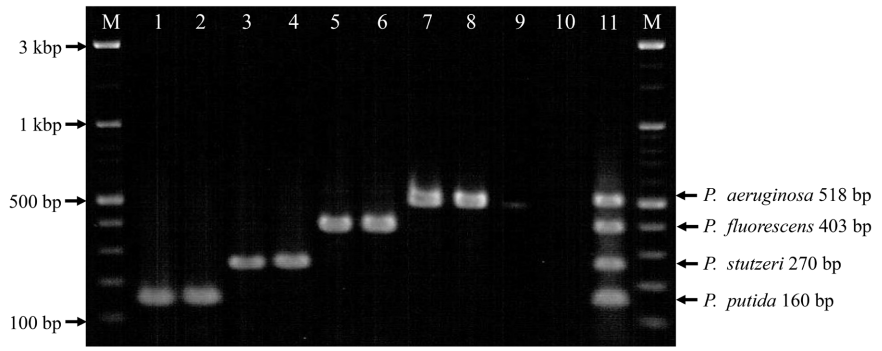

Figure 2. Specificity of the multiplex PCR assays for four *Pseudomonas* species. The primer mixture contained PPF, PPR, PSF, PSR, PFF, PFR, PAF and PAR. Lanes: 1, *Pseudomonas putida* JCM 6156; 2, *Pseudomonas putida* JCM 13063; 3, *Pseudomonas stutzeri* JCM 5965; 4, *Pseudomonas stutzeri* JCM 20778; 5, *Pseudomonas fluorescens* JCM 5963; 6, *Pseudomonas fluorescens* JCM 5965; 7, *Pseudomonas aeruginosa* JCM 5962; 8, *Pseudomonas aeruginosa* JCM 2776; 9, *Pseudomonas straminea* JCM 2783; 10, *Pseudomonas oleovorans* JCM 20560; 11, Mixture of ; *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* ;M, molecular size marker (100-bp DNA ladder).

Table 1. Detection frequency of four *Pseudomonas* species in fifty washstand samples.

	No. of positive samples (%) n = 50
Genus *Pseudomonas*	50 (100)
Pseudomonas putida	22 (44)
Pseudomonas aeruginosa	4 (8)
Pseudomonas stutzeri	2 (4)
Pseudomonas fluorescens	1 (2)

4. Discussion

P. aeruginosa is a ubiquitous bacterium normally found in water and soil, and also an opportunistic pathogen of humans, animals, and plants [13]. In humans, *P. aeruginosa* causes severe infections in patients with underlying conditions by nosocomial infection. Immunosuppressed or intubated-ventilated patients presenting compromised host defenses are particularly vulnerable to this pathogen. The simultaneous detection of *Pseudomonas* species other than *P. aeruginosa*, such as *P. stutzeri*, *P. fluorescens*, and *P. putida*, can be important since those organisms have sometimes been reported to be involved in opportunistic infections [14]. To develop a PCR-based technique more applicable for clinical use than conventional PCR, we established a multiplex PCR system for identifying and detecting simultaneously four medically important *Pseudomonas* species, using only one PCR tube per sample. A multiplex-PCR method is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents [15]. The most significant problem with regard to this method is the possibility of hybridization

among the different sequences of primers. A multiplex PCR for the detection of four medically important *Pseudomonas* species has not ever been developed. Therefore, a reliable identification method is needed to accurately assess the prevalence of four medically important *Pseudomonas* species, *i.e.*, *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens.*

The molecular detection and identification of microorganisms is widely used in microbiology and epidemiology. However, only limited information is now available on the molecular detection of microorganisms, and the development of appropriate strategies for their rapid identification and monitoring is needed. Some molecular approaches, such as ribotyping, PCR amplification of the 16S-23S rRNA gene spacer region, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, have been exploited for the analysis of the diversity of *Pseudomonas* isolates [16] [17] [18] [19]**.** The molecular identification of *Pseudomonas* species is often difficult and controversial. The sequence analysis of the 16S rRNA gene is widely employed for the identification of bacteria; however, this region is not satisfactorily discriminating between *Pseudomonas* species. Phylogenetic studies have highlighted that inferred phylogenies based on the 16S rRNA gene lack resolution at the intrageneric level because of its low rate of evolution [20]. Recent studies on the microbiota reported that it was difficult to achieve an unequivocal identification of *Pseudomonas* at the species level, even though variable regions of the 16S rRNA gene were analyzed [21]. Several authors have evaluated the use of alternative sequences for the identification and phylogenetic studies of Pseudomonas spp. For this purpose, the sequences of the *carA*, *recA*, *gyrB*, *fliC*, and *rpoD* genes of *Pseudomonas* species have been determined [22] [23] [24]. In the present study, the *rpoD* gene sequence was used as a target in order to design species-specific primers to selectively and simultaneously detect four medically important *Pseudomonas* species.

In the present study, we designed species-specific primers with the already mentioned means, for the identification of four medically important *Pseudomonas* species, *i.e.*, *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens* with a PCR method. These primers were able to distinguish each *Pseudomonas* species and did not display cross-reactivity with each other. Moreover, we developed a one-step multiplex PCR method with the ability to identify and differentiate four medically important *Pseudomonas* species using only each one PCR tubes per sample.

In the present study, the genus *Pseudomonas* was detected from all samples (100%), whereas *P. putida*, *P*, *aeruginosa*, *P. stutzeri* and *P. fluorescens* were detected at 44%, 8%, 4% and 2% in fifty swab samples, respectively. These results indicated that washstands were the uninhabitable environment for *P. putida*, *P. aeruginosa*, *P. stutzeri* and *P. fluorescens* and also these organisms detected from the human oral cavities were derived from the living environment other than the washstands.

Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction may be avoided, and the

subspecies identification and detection using this method only takes approximately 2 hours. Thus, using this method, the oral distribution of *P. putida*, *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens*, which were not analyzed in this study, and their involvement in various infections, will be fully clarified in future studies.

5. Conclusion

Our developed multiplex PCR method enables the reliable identification of four clinically important *Pseudomonas* species. Its simplicity means that it can be employed readily in most laboratories, where it might contribute to a better understanding of the epidemiology and clinical significance of the most important *Pseudomonas* species, *i.e.*, *P. putida*, *P*, *aeruginosa*, *P. stutzeri* and *P. fluorescens*.

Authors' Contributions

Hayashi S, Umezawa K, Tsuzukibashi O, Fukatsu A, Fuchigami M, Komine C, Yamamoto H, Hagiwara-Hamano M, Iizuka Y and Uchibori S corrected the data. Hayashi S, Umezawa K, Tsuzukibashi O, Fukatsu A, Wakami M, Murakami H, Kobayashi T, Fukumoto M and Nomoto T drafted and wrote the manuscript. The concept of this manuscript was devised by Fukatsu A. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest. No funding was received in this study.

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