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Study on Distribution of *Acinetobacter baumannii* Complex in Dental Hospital Using Multiplex PCR

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Abstract

Purpose: In recent years, multidrug-resistant *Acinetobacter baumannii* has appeared and caused outbreaks of hospital infections all over the world. Close monitoring of this pathogen and other *A. baumanii* complex species is considered of critical importance to public health organizations. The reliable identification method able to distinguish *A. baumannii* from genetically close *Acinetobacter* species is needed, because these species are unable to be differentiated by phenotypic or biochemical methods. The purpose of the present study was to design species-specific primers in order to identify and detect *A. baumanii* complex species, and *Acinetobacter lwoffii* which is frequently detected from the human specimens, and to investigate the distribution of these organisms in dental hospital using a multiplex PCR. **Methods:** Polymerase chain reaction (PCR) primers were designed based on partial sequences of the 16S ribosomal RNA (16S rRNA) gene and DNA gyrase subunit B (*gyrB*) of each species of *A. baumanii* complex species. Swab samples were collected from ten dental spittoon units in dental hospital, and the distribution of *A. baumanii* complex species was investigated using a multiplex PCR. **Results:** These primers were able to distinguish each species of *A. baumanii* complex species clearly. *A. baumanii* and *A. calcoaceticus* were detected at 20.0% and 10.0% in ten swab samples, respectively. On the other hand, *A. nosocomialis*, *A. lowffii*, and *A. pittii* were detected from no sample. **Conclusion:** Our de-

veloped one-step multiplex PCR method is accurate, specific, cost-effective, time-saving, and worked without requiring DNA extraction.

Keywords

Acinetobacter baumannii Complex, Multiplex PCR, Hospital Infections

1. Introduction

At present, the genus *Acinetobacter* comprises 108 species (https://lpsn.dsmz.de/genus/acinetobacter). An increasing incidence during the 1970s of resistant members of the family *Enterobacteriaceae* involved in nosocomial infections was followed by the therapeutic introduction of newer broad-spectrum antibiotics in hospitals and a subsequent increase in the importance of strictly aerobic gram-negative bacilli, including *Pseudomonas aeruginosa*, *Stenotrophomonas* (*Xanthomonas*) *maltophilia*, and *Acinetobacter* species.

Of these microorganisms, it is currently recognized that *Acinetobacter* species play an important role in the nosocomial infection. The genus *Acinetobacter* is now defined as including gram-negative coccobacilli, with a DNA G + C content of 39 to 47 mol%, that are strictly aerobic, nonmotile, catalase positive, and oxidase negative. These organisms have been implicated in a variety of nosocomial infections, including bacteremia, urinary tract infection, and secondary meningitis, but their predominant role is as agents of nosocomial pneumonia, particularly ventilator-associated pneumonia in patients confined to hospital intensive care units (ICUs). Such infections are often extremely hard for the clinician to treat because of the widespread resistance of these bacteria to the principal groups of antibiotics. Various mechanisms of antibiotic resistance have been recognized in these organisms, and combination therapy is usually required for effective treatment of *Acinetobacter* nosocomial infections. These therapeutic difficulties are coupled with the fact that these organisms have a significant capacity for long-term survival in the hospital environment, with corresponding enhanced opportunities for transmission between patients, either via human reservoirs or via medical materials.

It is currently considered that most clinically significant *Acinetobacter* species are *Acinetobacter baumannii*, *Acinetobacter nosocomialis*, and *Acinetobacter pittii*. These species are not differentiated by phenotypic or biochemical methods and are often grouped together diagnostically as the *A. baumannii* complex, along with *Acinetobacter calcoaceticus* which is generally not considered pathogenic. Species-level identification based on genetic methods (e.g., *rpoB* sequencing, rRNA intergenic spacer sequencing) has enabled differentiation of the *A. baumannii* complex species for research purposes. It has been demonstrated that matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) can be utilized as an alternative method to reliably identify *A. baumannii* complex species [1] [2] [3] [4]. Using these methods, the preva-

lence of *A. baumannii*, *A. nosocomialis*, and *A. pittii* within the *A. baumannii* complex has been shown to be highly variable among clinical isolates depending on the epidemiological settings [5] [6] [7] [8].

The detection of *A. baumannii* complex species 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 *A. baumannii* complex 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 *A. baumannii* complex 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 *A. baumannii* complex species is required.

The purpose of the present study was to design species-specific primers in order to identify and detect *A. baumanii* complex species, *i.e.*, *A. baumannii*, *A. nosocomialis*, *A. pittii* and *A. calcoaceticus*, and *Acinetobacter lwoffii* which is frequently detected from the human specimens, and to investigate the distribution of these organisms in the dental hospital 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) and Center for Conservation of Microbial Genetic Resource, Gifu University (GTC; Japan). The following bacterial strains were used in the present study: *A. baumannii* JCM 6841, *A. calcoaceticus* JCM 6842, *A. lwoffii* JCM 6840, *A. pittii* GTC 00524, and *A. nosocomialis* GTC 03314. 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 *A. baumanii* Complex Species and *A. lwoffii*

Design of species-specific primers for *A. baumanii* complex species, *i.e.*, *A. baumannii*, *A. nosocomialis*, *A. pittii* and *A. calcoaceticus*, and *A. lwoffii* was performed as described previously [9]. Briefly, the 16S rRNA gene sequences of *A. baumannii* (accession no. AB594765), *A. nosocomialis* (HQ180192), *A. pittii* (MN307289), *A. calcoaceticus* (AB626122) and *A. lwoffii* (X81665), and the DNA gyrase subunit B (*gyrB*) gene sequences of *A. baumannii* (AB594765), *A. nosocomialis* (HQ180192), *A. pittii* (MN307289), *A. calcoaceticus* (AB626122) and *A. lwoffii* (X81665) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple se-

quence alignment analysis was performed with the CLUSTAL W program; *i.e.*, the 16S rRNA and *gyrB* gene sequences of four *A. baumanii* complex species and *A. lwoffii* were aligned and analyzed, respectively. Homology among the primers selected for each *Acinetobacter* species and their respective 16S rRNA and *gyrB* 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 re-suspended 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 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, 62˚C for 15 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. Environmental Samples

Swab samples were collected from ten dental spittoon units in a dental hospital (Nihon University Hospital, School of Dentistry at Matsudo), and the distribution of *A. baumanii* complex species and *A. lwoffii* in the dental hospital environment was investigated using a multiplex PCR. Swab samples which wiped the sink drain traps of each dental spittoon unit were collected in a sterile microcentrifuge tube. 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 16S rRNA and *gyrB* gene sequences of four *A. baumanii* complex species and *A. lwoffii* were designed in the present study (**Figure 1** and **Figure 2**). The specific forward primers were designated as ABF for *A. baumannii*, ACF for *A. calcoaceticus*, ANF

for *A. nosocomialis*, APF for *A. pittii*, and ALF for *A. lwoffii*, whereas the specific reverse primers were designated as ABR for *A. baumannii*, ACR for *A. calcoaceticus*, ANR for *A. nosocomialis*, APR for *A. pittii*, and ALR for *A. lwoffii*. The amplicon sizes of *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii*, and *A. lwoffii* were 564 bp, 397 bp, 731 bp, 105 bp, and 274 bp, respectively.

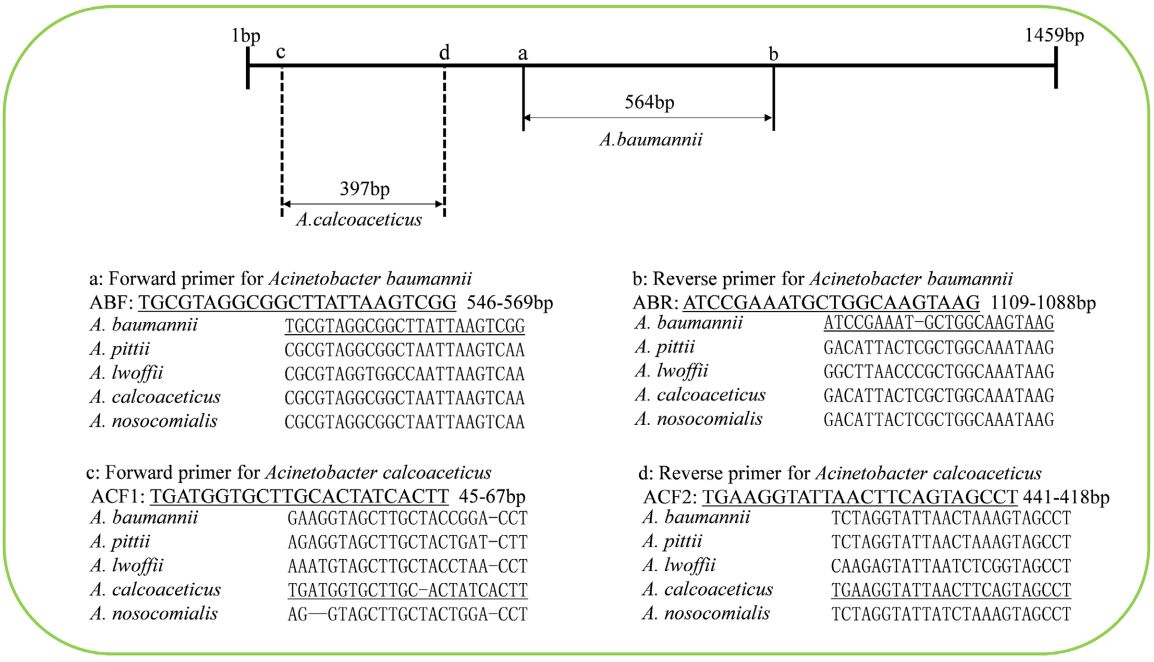

Figure 1. Species-specific primers for 16S rRNA gene of *A. baumannii* and *A. calcoaceticus*. The nucleotide sequence of each primer has been underlined.

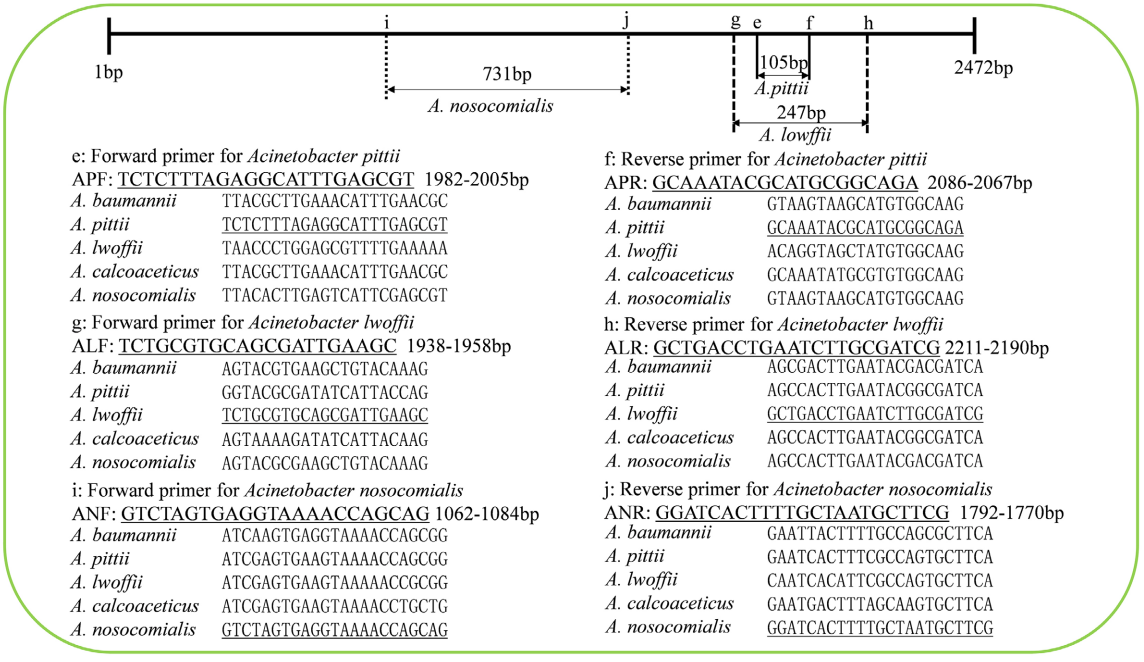

Figure 2. Species-specific primers for the *gyrB* gene of *A. pittii*, *A. lwoffii* and *A. nosocomialis*. The nucleotide sequence of each primer has been underlined.

3.2. Multiplex PCR

Our multiplex PCR method for identifying and detecting five *Acinetobacter* species, *i.e.*, *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii*, and *A. lwoffii* successfully amplified DNA fragments of the expected size for each species (Figure 3). 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 F *A. baumannii*-specific primer set with strain JCM 6841, the *A. calcoaceticus*-specific primer set with strain JCM 6842, the *A. nosocomialis*-specific primer set with strain GTC 03314, the *A. pittii*-specific primer set with strain GTC 00524 and the *A. lwoffii*-specific primer set with strain JCM 6840 (data not shown).

3.3. Distribution of *A. baumanii* Complex Species and *A. lwoffii* in Dental Hospital Environment

Table 1 shows the detection frequency of five *Acinetobacter* species in ten dental units. *A. baumanii* and *A. calcoaceticus* were detected at 20.0% and 10.0% in ten swab samples, respectively. Among them, two *Acinetobacter* species were found from one unit. On the other hand, *A. nosocomialis*, *A. lowffii*, and *A. pittii* were detected from no sample.

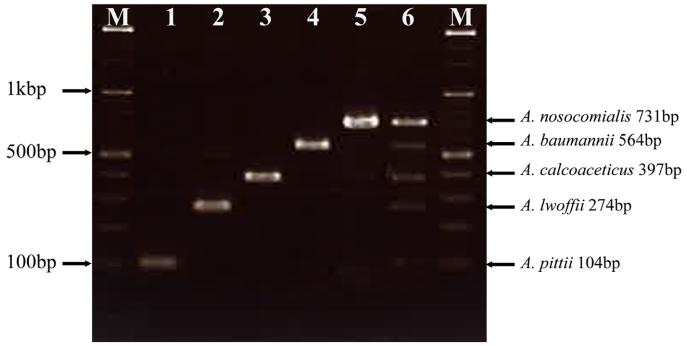

Figure 3. Specificity of the multiplex PCR assays for *A. baumanii* complex species and *A. lwoffii*. The primer mixture contained ABF, ABR, ACF, ACR, ANF, ANR, APF, APR, ALF and ALR. Lanes: 1: *A. pittii* GTC 00524; 2: *A. lwoffii* JCM 6840; 3: *A. calcoaceticus* JCM 6842; 4: *A. baumannii* JCM 6841; 5: *A. nosocomialis* GTC 03314; 6: Mixture of *A. pittii*, *A. lwoffii*, *A. calcoaceticus*, *A. baumannii* and *A. nosocomialis*. M, molecular size marker (100-bp DNA ladder).

Table 1. Detection frequency of five *Acinetobacter* species in ten dental units.

	No. of positive samples n = 10 (Frequency, %)
A. baumannii	2 (20)
A. nosocomialis	0 (0)
A. calcoaceticus	1 (10)
A. lwoffii	0 (0)
A. pittii	0 (0)

4. Discussion

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 five medically important *Acinetobacter* 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 [10]. The most significant problem with regard to this method is the possibility of hybridization among the different sequences of primers. Higgins *et al.* reported a PCR strategy allowing the identification of four *A. baumanii* complex species as same as the present study [11]. However, the findings of our pilot study showed that the size of each PCR fragment by this method was similar; therefore, it was difficult to accurately identify each *A. baumanii* complex species. Moreover, including DNA extraction, it took more than 3 hours to finish the identification. Therefore, a reliable identification method is needed to accurately assess the prevalence of *A. baumanii* complex species.

Identification of *Acinetobacter* isolates to the species level is often difficult, especially in routine diagnostic laboratories [12]. The clinically relevant species *A. baumannii*, *A. nosocomialis* (formerly genomic species 13TU) and *A. pittii* (formerly genomic species 3) are often grouped together alongside the environmental *A. calcoaceticus* species as *A. baumannii* complex because they are genetically closely related and phenotypically very difficult to differentiate from each other [13]. However, there are considerable epidemiological and clinically relevant differences among these species. *A. calcoaceticus* is an environmental organism that, to our knowledge, has never been involved in serious human disease, and therefore it should not be misidentified as *A. baumannii*. The natural habitats of *A. baumannii* and *A. nosocomialis* are unknown, as are the differences in their epidemic behaviors, resistance mechanisms, and pathogenicities. *A. pittii* can be found regularly on human skin, as well as in aquatic environments [14]. *A. pittii* has also been implicated in nosocomial infections, but its tendency for epidemic spread and resistance development is far less pronounced than that of *A. baumannii* [14] [15]. For epidemiological and clinical purposes, it is therefore highly desirable to differentiate among these species correctly.

In the present study, we designed species-specific primers with the already mentioned means, for the identification of medically important *A. baumanii* complex species, *i.e.*, *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii*, and *A. lwoffii* with a PCR method. These primers were able to distinguish each *Acinetobacter* 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 *A. baumanii* complex species and *A. lwoffii* using only each one PCR tubes per sample. Species-specific primers for five medically important *Acinetobacter* species were designed based on the sequences of 16S rRNA and *gyrB* genes. The protein-coding genes that have been tested for the

assessment of microbial diversity include the genes for DNA gyrase subunit B (*gyrB*) [16], RNA polymerase subunit B (*rpoB*) [17], the TU elongation factor (*tuf*) [18], the 60 kDa chaperonin protein (*cpn60*) [19]. The *gyrB* gene has a number of potential advantages. Previous reports have shown these markers to be suitable for phylogenetic analyses, as it provides a better resolution at species level than the 16S rRNA gene [20].

In the present study, *Acinetobacter* species were detected from only two of ten dental spittoon units in a dental hospital. Numerous studies have documented the presence of *Acinetobacter* species in the hospital environment, but rates of positive cultures may vary widely, depending on the epidemiological setting. In the previous study, *Acinetobacter* species have been found in 27% of hospital sink traps and 20% of hospital floor swab cultures [21]. The result of the present study was similar to that of the previous study.

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, the method described herein will allow the prevalence of *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii* and *A. lwoffii* and their involvement in the various infections, to be fully clarified in future studies.

5. Conclusion

Our developed multiplex PCR method enables the reliable identification of the clinically most relevant *Acinetobacter* 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 *Acinetobacter* species, *i.e.*, *A. baumanii* complex species.

Authors' Contributions

Fukatsu A, Tsuzukibashi O, Yamamoto H, Takahashi Y, Usuda K, Fuchigami M, Uchibori S, Komine C, Umezawa K, Hayashi S and Asano T corrected the data. Fukatsu A, Tsuzukibashi O, Wakami M, Murakami H, Kobayashi T and Fukumoto M 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.

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