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Study on Distribution of *Acinetobacter baumannii* in Human Oral Cavity and Dental Hospital Using Culture Method

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Abstract

Purpose: In recent years, outbreaks of multidrug-resistant *Acinetobacter baumannii* have become a significant concern, making the monitoring of this bacterium an urgent priority. Therefore, there is an urgent need to establish a simple and highly specific method for its isolation and identification. The objective of this study was to develop a selective medium for isolating *A. baumannii* and to investigate the contamination status of this organism in the human oral cavity and within dental hospitals. **Methods:** The base medium showing the best growth was supplemented with antibiotics that did not inhibit the growth of this organism, resulting in a selective medium for detecting *A. baumannii*. Subsequently, we attempted to detect *A. baumannii* using the developed selective medium for isolation, followed by identification via PCR. Samples included 30 human saliva specimens and swabs taken from the drain traps of 30 dental units used in clinical practice. We also investigated the multidrug resistance tendencies of the isolated *A. baumannii* strains. **Conclusion:** Using the isolation method with this selective medium and PCR-based identification, *A. baumannii* was detected from four dental units, but not from any saliva samples. Furthermore, no multidrug-resistant *Acinetobacter baumannii* was detected at all. This method is presumed to be useful for investigating the distribution of *A. baumannii* in various samples.

Keywords

Acinetobacter baumannii, Selective Medium, Oral Cavity

1. Introduction

Acinetobacter species are bacteria found in environments such as soil and water systems. Although Gram-negative, these bacteria are known to survive long-term not only in moist environments but also on dry surfaces. They are typically considered non-pathogenic, but they can colonize human skin and mucous membranes, causing opportunistic infections in immunocompromised patients. Therefore, in healthcare facilities, nosocomial infections such as pneumonia, urinary tract infections, and catheter-associated bloodstream infections are problematic [1]-[3].

At present, the genus *Acinetobacter* comprises 122 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. Among the genus *Acinetobacter*, *A. baumannii* is considered clinically the most significant. However, because it is difficult to distinguish it from the three species *A. nosocomialis*, *A. pittii*, and *A. calcoaceticus*, which share very similar basic characteristics, it is collectively referred to as the *A. baumannii* complex [4]. Other known species include *A. lwoffii*, *A. radioresistens*, *A. ursingii*, and *A. junii*. For routine identification of *Acinetobacter* species, rapid identification kits and automated instruments are used. However, compared to enterobacteria, *Acinetobacter* species exhibit limited biochemical characteristics, and the species identifiable by each rapid kit vary significantly. Consequently, the accuracy of identification when using these methods alone has limitations.

In recent years, the spread and increase of multidrug-resistant Gram-negative bacilli, which have acquired resistance to multiple antibiotics including carbapenems, aminoglycosides, and fluoroquinolones, has become a global problem [5] [6]. Among these, carbapenem-resistant Enterobacteriaceae (CRE), multidrug-resistant *Pseudomonas aeruginosa* (MDRP), and multidrug-resistant *Acinetobacter* species (MDRA) [7] are particularly significant not only for infection treatment but also from the perspective of infection control. In Japan, following the 2008 report of an MDRA outbreak at a university hospital in Fukuoka Prefecture triggered by an importation from South Korea, subsequent outbreaks occurred at university hospitals in Tokyo in 2009 and Aichi Prefecture in 2010, bringing MDRA and other multidrug-resistant Acinetobacter species into sharp focus.

Detecting *A. baumannii* in clinical specimens is important as it can influence prognosis and patient management; however, identification using conventional biochemical methods can be difficult. Accurate identification and quantification of *A. baumannii* is necessary to elucidate its role in various systemic diseases. These microorganisms can be identified through sequence analysis of multiple target genes or MALDI-TOF mass spectrometry [8]-[11]. However, these methods are cumbersome, expensive, and time-consuming, making them unsuitable for detecting and differentiating *A. baumannii* in clinical isolates. Consequently, epidemiological studies investigating the association between these microorganisms and various diseases remain limited. Therefore, a simple and reliable test for identifying *A. baumannii* is needed. Furthermore, selective media are necessary to examine the biological characteristics and antimicrobial resistance patterns of *A. baumannii* strains detected from clinical specimens; however, selective media for isolating *A. baumannii* have yet to be developed.

The objectives of this study are to develop selective media for isolating *A. baumannii* and an identification method using PCR, and to investigate the contamination status of this bacterium in the human oral cavity and within dental clinics.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Bacterial strains were obtained from Japan Collection of Microorganisms (JCM; Japan), Center for Conservation of Microbial Genetic Resource, Gifu University (GTC; Japan), and American Type Culture Collection (ATCC; America). All bacterial strains used in the present study are listed in **Table 1**. Bacterial strains used in the present study 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.

Table 1. Recovey of *A. baumannii* and other bacteria on BHI agar and ABSM.

Species	Strain	BHI-Y CFU/ml, $\times 10^8$	ABSM CFU/ml, $\times 10^8$	Recovery, %
Acinetobacter baumannii	JCM 6841	1.6 ± 0.2[a]	1.6 ± 0.3	99.3
	GTC 03319	0.8 ± 0.3	0.8 ± 0.2	95.5
	GTC 14637	1.1 ± 0.3	1.1 ± 0.1	98.0
	Num-6001	2.1 ± 0.4	2.0 ± 0.2	97.1
	Num-6002	1.1 ± 0.3	1.0 ± 0.3	97.5
Acinetobacter nosocomialis	GTC 03314	3.3	0	0
Acinetobacter calcoaceticus	JCM 6842	3.3	0	0
Acinetobacter lowffii	JCM 6840	3.3	0	0
Acinetobacter pittii	GTC 12034	3.3	0	0

Continued

Klebsiella pneumoniae	ATCC 13883	1.7	0	0
Raoultella planticola	DSM 3069	2.1	0	0
Citrobacter freundii	DSM 30039	3.9	0	0
Serratia marcescens subsp. *marcescens*	JCM 1239	0.7	0	0
Serratia marcescens subsp. *sakuensis*	JCM 11315	0.8	0	0
Pseudomonas aeruginosa	JCM 5962	6.3	0	0
Pseudomonas fluorescens	JCM 5963	3.1	0	0

[a]Ave ± SD.

2.2. Development of New Selective Medium

2.2.1. Evaluation of Base Medium

BHI agar supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHI-Y blood), Nutrient agar (NA), and CVT agar (Shimadzu Diagnostics Co., Tokyo, Japan) were examined as the base medium in the selective medium. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates inoculated with bacteria were cultured at 30˚C for 48 h under an aerobic condition. After cultivation, the number of colony-forming units (CFU)/ml was counted.

2.2.2. Susceptibility Tests

Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing [12].

2.3. Recovery of *Acinetobacter* Species and Other Representative Bacteria

The recoveries of the *Acinetobacter* reference strains and other representative bacteria were calculated as CFU/ml on selective medium and compared with those on BHI agar for total cultivable bacteria. All bacterial strains used in the present study are listed in Table 1.

All bacterial strains were pre-incubated in BHI broth at 30˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates inoculated with bacteria were cultured at 30˚C for 48 h under an aerobic condition. After cultivation, the number of CFU/ml was counted.

2.4. Clinical Samples

Thirty volunteers (15 men, 15 women; mean age 38 years, range 15 - 71 years)

participated in the present study. They had no systemic disease and received no antibiotic therapy for at least 3 months. All participants were asked not to brush, rinse, or smoke immediately prior to the assessment and not to eat or drink for at least 2 h beforehand.

Paraffin-stimulated whole saliva samples were collected in a sterile microcentrifuge tube. Swab samples were collected from ten dental spittoon units in a dental hospital (Nihon University Hospital, School of Dentistry at Matsudo). 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 0.1 ml of each was diluted and inoculated on BHI-Y and selective medium plates. BHI-Y plates for total cultivable bacteria were cultured at 37˚C for 2 days in an atmosphere of 5% CO_2 in a CO_2 incubator, and selective medium plates for *A. baumanii* were cultured at 30˚C for 2 days under an aerobic condition. After cultivation, CFU/ml in each sample was calculated. The present study was conducted in accordance with the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC23-012).

2.5. Identification of *A. baumanii* Isolated from Clinical Samples

For each sample, 24 colonies were randomly selected from approximately 50 colonies that had grown on selective agar plates. Each selected colony was completely isolated and subcultured by streaking onto BHI-Y medium. Subsequently, bacterial species identification was performed using PCR analysis.

2.6. Design of Species-Specific Primers for *A. baumanii*

Design of species-specific primers for *A. baumanii* was performed as described previously [13]. 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) 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 16S rRNA gene sequences of five *Acinetobacter* species were aligned and analyzed, respectively. Homology among the primers selected for each *Acinetobacter* species and their respective 16S rRNA sequences was confirmed by a BLAST search.

2.7. Development of 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 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.

2.8. Antimicrobial Susceptibility of Isolated *A. baumanii* Strains

The screening of antimicrobial susceptibility of isolated *A. baumanii* strains was performed by disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [14]. In total, the used antimicrobial agents included amikacin (30 μg), imipenem (10 μg), and levofloxacin (5 μg) (Sensi-Disk, Becton Dickinson Co., MD, USA). MDRA were defined as strains resistant to all agents in three antimicrobial categories. The susceptibility testing criteria for three antimicrobial agents were set as follows: For amikacin, an inhibition zone diameter of less than 14 mm; For imipenem, an inhibition zone diameter of less than 13 mm; For levofloxacin, an inhibition zone diameter of less than 13 mm.

3. Results

3.1. Development of Selective Medium

3.1.1. Selection of Base Medium

The selection of a base medium for the growth of *A. baumanii* was performed. *A. baumanii* grew well on CVT agar as same as BHI-Y, BHI-Y blood, and NA (data not shown). To inhibit the growth of Gram-positive bacteria, CVT was ultimately selected as the base medium.

3.1.2. Susceptibility to Antibiotics

A. baumanii was more resistant to chloramphenicol than the genus *Klebsiella*. The minimal inhibitory concentration (MIC) of chloramphenicol for *A. baumanii* was more than 40 μg/ml. *A. baumanii* was more resistant to aztreonam than *Escherichia coli*. The MIC of aztreonam for *A. baumanii* was 20 μg/ml. The genus *Microbacterium* was sensitive to 5 μg/ml of lincomycin. The MIC of lincomycin for *A. baumanii* was 20 μg/ml. *A. baumanii* was more resistant to sodium deoxycholate than *Pseudomonas*, *Raoultella*, *Citrobacter*, and *Serratia* species. The MIC of sodium deoxycholate for *A. baumanii* was 1000 μg/ml. The genus *Pseudomonas* were sensitive to 1 μg/ml of sodium deoxycholate. *A. baumanii* was more resistant to cefsulodin than the genus *Staphylococcus*. The MIC of cefsulodin for *A. baumanii* was 25 μg/ml. The genus *Staphylococcus* and *Acinetobacter* species exclud-

ing *A. baumanii* were sensitive to 6 μg/ml of cefsulodin.

3.1.3. Composition of New Selective Medium

The new selective medium, designated oral *A. baumanii* selective medium (ABSM), was composed of the following (per liter): 23.5 g of CVT agar, 15 mg of chloramphenicol, 3 mg of aztreonam, 5 mg of lincomycin, 250 mg of sodium deoxycholate, 6 mg of cefsulodin, and 15 mg of amphotericin B. Antibiotics, *i.e.*, chloramphenicol, aztreonam, lincomycin, sodium deoxycholate cefsulodin, and amphotericin B were added after the base medium had been sterilized and cooled to 50˚C.

3.1.4. Recovery of *A. baumanii* and Inhibition of Other Representative Bacteria on Selective Medium

Table 1 shows the recovery of some *A. baumannii* reference strains on ABSM relative to BHI-Y. The growth recoveries of the *A. baumannii* reference strains on ABSM were between 97.5% and 99.3% (average 97.5%) that on BHI-Y.

Table 1 also shows the inhibition of other representative bacteria except *A. baumannii* on ABSM relative to BHI-Y. The growth of other representative bacteria was markedly inhibited on the selective medium.

3.2. PCR Method for Identifying *A. baumanii*

3.2.1. Primer Design

The specific primer set covering the upstream region of the 16S rDNA sequence of *A. baumannii* was designed in the present study (Table 2). The amplicon size of *A. baumanii* was 562 bp.

Table 2. Locations and sequences of species-specific primers for the16S rDNA of *A. baumannii*.

Primer	Sequence	Product size (bp)	Position	Accession number
ABF	CGTAGGCGGCTTATTAAGTCGG	562	129 - 148	AB594765
AIR	ATCCGAAATGCTGGCAAGTAAG		1035 - 1015	

3.2.2. PCR Condition

A PCR method for identifying *A. baumannii* successfully amplified DNA fragments of the expected size (Figure 1). 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 *A. baumannii*-specific primer set with strain JCM 6841 (data not shown).

3.2.3. Assay of *A. baumannii* and Representative Oral Bacteria

The PCR method used to identify *A. baumannii* produced positive bands from the *A. baumannii* reference strain JCM 6841 and GTC 03319 (Figure 1). No amplicons were produced from any of the representative oral bacteria and microorganisms colonizing the environment (data not shown).

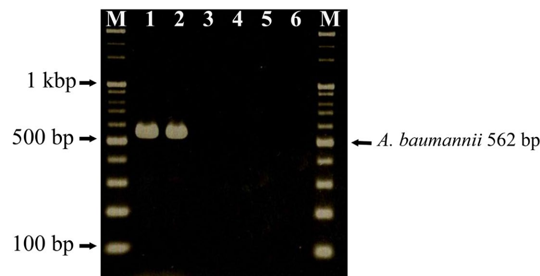

Figure 1. Specificity of PCR assays for *A. baumanii*. The primer mixture contained ABF and ABR. Lanes: 1, *A. baumannii* JCM 6841; 2, *A. baumannii* GTC 03319; 3, *A. calcoaceticus* JCM 6842; 4, *A. pittii* GTC 00524; 5, *A. nosocomialis* GTC 03314; 6, *A. lwoffii* JCM 6840. M, molecular size marker (100-bp DNA ladder).

3.3. Clinical Examination

The detection frequencies of *A. baumanni* in the saliva samples from thirty healthy subjects and the swab samples from thirty dental spittoon units are shown in Table 3. *A. baumannii* was not detected at all in the saliva samples (0%). On the other hand, this microorganism was detected from nine swab samples. In positive samples, the mean number of this microorganism and its proportion relative to the total bacterial number were 1.10×10^4 CFU/ml and 0.065%, respectively. MDRA was not detected in any of the samples.

Table 3. Detection frequency of *A. baumannii* in the human oral cavity and dental units.

	No. of A. baumannii positive samples (%, frequency)	No. of MDRA positive samples (%, frequency)	No. of total bacteria $\times 10^8$ CFU/ml	No. of *A. baumannii* $\times 10^4$ CFU/ml (%, *A. baumannii*/ total bacteria)
Human oral cavity (n = 30)	0 (0)	0 (0)	1.68	0 (0)
Dental units (n = 30)	9 (30)	0 (0)	0.17	1.10 (0.065)

In the first isolation, *A. baumannii* colonies on ABSM commonly had a smooth appearance resembling a fried egg. The colony's color was reddish purple at the center, with white surrounding it. Therefore, ABSM could distinguish from other bacteria based on colony morphology. The average colony sizes of *A. baumannii* on ABSM were 2.1 mm in diameter (Figure 2).

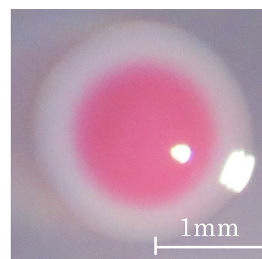

Figure 2. Appearance of *A. baumanii* colonies on ABSM.

4. Discussion

Identification of *Acinetobacter* isolates to the species level is often difficult, especially in routine diagnostic laboratories [15]. 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 [16]. 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 [17]. *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* [17] [18]. For epidemiological and clinical purposes, it is therefore highly desirable to differentiate among these species correctly.

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 [19]. 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 [20]. 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*.

In the present study, we designed species-specific primers with the already mentioned means, for the identification of medically important *A. baumanii* with a PCR method. Species-specific primers for this microorganism were designed based on the sequences of 16S rRNA gene. These primers were able to distinguish *A. baumanii* with others and did not display cross-reactivity with those. Our 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.

A useful selective medium for isolating *A. baumanii* may contribute to the correct and rapid diagnosis of infectious diseases caused by this organism. However, a selective medium that is useful for the isolation of *A. baumanii* has not ever been developed. In the present study, *A. baumanii* strains were more resistant to sodium fluoride, ofloxacin, fosfomycin, and colistin than other representative bacteria. The growth of other representative bacteria and fungi was inhibited by the addition of 15 mg of chloramphenicol, 3 mg of aztreonam, 5 mg of lincomycin,

250 mg of sodium deoxycholate, 6 mg of cefsulodin, and 15 mg of amphotericin B to CVT agar. All of the *A. baumanii* reference strain and isolates tested grew well on the new selective medium, designated as ABSM, while the growth of other bacteria was markedly inhibited (**Table 1**). Moreover, ABSM allowed for the identification of *A. baumanii* by its characteristic colony morphology.

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. *Acinetobacter* species have also been found occasionally in the oral cavity and respiratory tract of healthy adults [22] [23], but the carriage rate of *Acinetobacter* species in nonhospitalized patients, apart from on the skin, is normally low [1]. In the present study as well, *A. baumannii* was not detected at all in the saliva samples from healthy subjects. *A. baumannii* might be not a part of the normal oral flora.

We developed selective medium ABSM to isolate *A. baumannii* from various specimens. ABSM exhibits high selectivity for *A. baumannii*, making it useful for evaluating the distribution and role of this microorganism in humans and various environmental locations.

5. Conclusion

The selective medium (ABSM) and our PCR method as isolation and identification methods, respectively, for *A. baumanii* may contribute to a better understanding of the epidemiology and clinical significance of the most important *Acinetobacter* species, *i.e.*, *A. baumanii*.

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Authors' Contributions

Fukatsu A, Tsuzukibashi O, Tayama T, Idei K, Usuda K, Uchibori S, Umezawa K, Iizuka Y 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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