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Original Research

Molecular epidemiological analysis of drug-resistant *Acinetobacter* spp. strains detected in a northern Osaka Prefecture medical facility

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Abstract

In recent years, the spread of drug-resistant bacteria has become a serious global problem. *Acinetobacter* species, a group of pathogens that commonly cause opportunistic infections, have become increasingly drug resistant, and cases of healthcare-associated infections have been reported.

In this study, we characterized 16 drug-resistant *Acinetobacter* spp. strains detected at a medical facility in northern Osaka Prefecture in 2019 and 2020, through molecular epidemiological analyses. Although a multidrug-resistant *Acinetobacter* strain that showed resistance to the three classes of drugs, namely carbapenems, aminoglycosides, and new quinolones, was not detected; seven strains were found to be resistant to two drugs. Moreover, species and strain identification using the PCR-based ORF typing (POT) method revealed that 15 of the strains were *A. baumannii* and three were international clones known to cause outbreaks. However, the POT method could not identify one strain, which we later determined as *A. dispersus* using 16S rRNA gene sequencing analysis. Our results indicate that the number of detected strains and POT types has increased and diversified over time. In addition, some clonal strains with the same POT value showed expansion of drug resistance, suggesting that trends in drug resistance must continue to be closely monitored.

Key words: *Acinetobacter*, MDRA, drug resistance, POT method

I. Introduction

In recent years, the spread of drug-resistant bacteria has become a serious global problem. The World Health Organization (WHO) published the "Global Action Plan," which calls for member countries to formulate action plans for raising awareness and providing education regarding drug-resistant microorganisms as well as surveillance and infection prevention and control[1]. In response, Japan enacted various measures, including the "Drug Resistance Action Plan" in 2016[2].

The genus *Acinetobacter* is a non-fermenting gram-negative bacillus widely found in nature, mainly in the pedosphere and hydrosphere. To date, 96 *Acinetobacter* species have been identified[3]. *A. baumannii* is a clinically important species that has been isolated from hospital environments and skin of healthcare workers. *A. baumannii* is usually harmless and non-pathogenic in healthy people; however, it is a typical cause of opportunistic infections, such as pneumonia, urinary tract infections, and septicemia, in immunocompromised patients[4]. While other glucose non-fermenting gram-negative bacilli prefer to inhabit a moist environment and cannot survive in dry conditions for a long period, the genus *Acinetobacter* is characterized by its ability to survive for several weeks, even in dry conditions, making it more likely to cause healthcare-associated infections[4].

In Japan, multidrug-resistant *Acinetobacter* (MDRA) is defined as a strain resistant to the three classes of drugs, namely carbapenems, aminoglycosides, and new quinolones. Since 2014, MDRA infection has been classified as a category V infectious disease under the Infectious Diseases Law. According to public information from the Nosocomial Infection Control Surveillance Project of the Ministry of Health, Labour, and Welfare[5], the aggregated results from the testing department showed that the isolation rate of MDRA has remained below 0.01% for over the past 5 years. However, in 2019, 19 cases of nosocomial infection caused by MDRA were reported at a medical facility in eastern Osaka[6]. MDRA is frequently isolated as a pathogen causing artificial respiration-associated pneumonia, catheter infections, and wound infections; however, its dynamics in medical facilities are not well understood[7]. As the spread of infection can occur through various routes, it is important to evaluate the spread of clonal strains in patients. In this study, we aimed to analyze the dynamics of the drug-resistant *Acinetobacter* spp. clinical isolates through strain identification using a molecular epidemiological method[8].

Ⅱ. Materials and Methods

1) Bacterial isolates and growth conditions

The target strains were drug-resistant *Acinetobacter* spp. with resistance to any of the following: doripenem (DRPM), amikacin (AMK), and levofloxacin (LVFX). The strains were isolated from different patients between January 2019 and December 2020 at a mixed-care medical facility located in northern Osaka Prefecture with approximately 1.000 beds, the majority of which were for patients requiring long-term care. The strains were cultured in Mueller-Hinton (MH) medium containing appropriate antibiotics at 37 ℃ for 12 h.

2) Drug susceptibility testing

The susceptibility of target strains to sulbactam/ampicillin (S/A), piperacillin (PIPC), tazobactam/piperacillin (T/P), ceftazidime (CAZ), meropenem (MEPM), DRPM, AMK, minocycline (MINO), and LVFX was assessed using the MicroScan WalkAway *plus* system (Beckman Coulter, Brea, USA). Neg EN Combo 1J was used for the drug panel, and the target strains were classified as susceptible (S), intermediate (I), or resistant (R) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

3) Discrimination of *A. baumannii* strains using PCR-based ORF typing method

The PCR-based ORF typing (POT) method, which can identify and distinguish between isolates at the strain level, is based on multiplex PCR. The target genomic regions contain species-specific genes and genomic islets[9]. The output is determined by the presence or absence of the target DNA regions in the genome and reflects the homogeneity between strains. *A. baumannii* strains were discriminated by the POT method using the Cica Geneus Acineto POT kit (Kanto Chemical, Tokyo, Japan) containing Apta Taq DNA polymerase. The genomic DNA of the target strains were extracted using Cica Geneus DNA Extraction Reagent (Kanto Chemical). The specific target regions of the extracted DNA were amplified using multiplex PCR and visualized using 4% agarose gel electrophoresis. To identify each strain, PCR product patterns were categorized into three groups based on their quantified POT values.

4) 16S rRNA gene sequencing analysis

The POT method used in this study can also identify *A. baumannii*, *A. pittii*, *A. nosocomialis*, and other *Acinetobacter* species close to *A. nosocomialis*[9]. Strains that could not be identified using the POT method were determined using DNA sequence analysis of the 16S rRNA gene. Genomic DNA was obtained using Cica Geneus DNA Extraction Reagent, which was used to amplify the 16S rRNA gene using PCR according to the method described by Liu and colleagues[10]. The obtained PCR products were purified using the Agencourt AMpure XP kit (Beckman Coulter), and the DNA sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific, Waltham, USA) and ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Waltham, USA). Homology searches of the DNA sequences were performed using BLASTn[11].

Ⅲ. Results

1) Drug susceptibility of the drug-resistant *Acinetobacter* strains

Sixteen strains of drug-resistant *Acinetobacter* spp. were isolated via drug susceptibility testing (Table 1). Eleven strains were resistant to the carbapenem DRPM, and 12 strains were resistant to the new quinolone LVFX. All the strains were sensitive to the aminoglycoside AMK. Therefore, although we did not detect MDRA strains that were resistant to all three drug classes, seven strains were resistant to two of the drug classes.

2) Strain discrimination using POT method

Analysis using the POT method showed that 15 of the 16 strains were *A. baumannii*, which were then classified into seven types through strain discrimination (Table 1). In addition, four groups of strains, including multiple clonal strains with the same POT values, were detected.

3) Species identification of the AUH-361 strain

PCR amplification of the 16S rRNA gene of the AUH-361 strain, which could not be identified using the POT method, generated a 1,404 bp DNA fragment. BLASTn analysis of this DNA sequence revealed 99.86% homology with *Acinetobacter dispersus*.

Ⅳ. Discussion

In this study, we conducted drug susceptibility testing and molecular epidemiological analysis of 16 drug-resistant *Acinetobacter* spp. strains collected at a medical facility in northern Osaka in 2019 and 2020. Well-known methods for strain discrimination based on genetic analysis, such as pulsed-field gel electrophoresis and multi-locus sequence typing (MLST), were performed[12]. In contrast, the POT method used in this study discriminates between strains by amplifying specific gene regions of the target strains via multiplex PCR and quantifying the PCR products via agarose gel electrophoresis. This method is characterized by a relatively simple testing process and an easy numerical comparison with other regionally detected strains.

Our analysis identified 15 of the 16 strains as *A.*

Table 1 Antibacterial susceptibility tests and strains clssifications by the POT method of *Acinetobacter species*

Strain	Breakpoint									POT number			Period
	S/A	PIPC	T/P	CAZ	MEPM	DRPM	AMK	MINO	LVFX	POT-1	POT-2	POT-3	
AUH-352	R	S	S	S	S	S	S	S	R	**122**	**35**	**50**	
AUH-353	R	S	S	S	S	S	S	S	R	**105**	**12**	**20**	
AUH-354	R	S	S	S	S	S	S	S	R	**105**	**12**	**20**	
AUH-359	R	S	S	**R**	S	S	S	S	R	**122**	**35**	**50**	2019. 01～2019. 12
AUH-360	R	S	S	S	S	**R**	S	S	R	**105**	**12**	**20**	
AUH-361	R	**I**	S	S	S	R	S	S	S	—	—	—	
AUH-363	R	S	S	S	S	**R**	S	S	R	**122**	**35**	**50**	
AUH-366	R	S	S	S	S	R	S	S	S	**92**	**11**	**0**	
AUH-369	R	S	S	S	S	R	S	S	S	**104**	**12**	**18**	
AUH-371	R	S	S	S	S	R	S	S	S	**104**	**12**	**20**	
AUH-372	R	S	S	S	S	**R**	S	S	R	**105**	**12**	**20**	
AUH-374	R	S	S	S	S	S	S	S	R	**105**	**12**	**20**	2020. 01～2020. 12
AUH-375	R	S	S	S	S	R	S	S	R	**105**	**12**	**4**	
AUH-376	R	S	S	S	S	R	S	S	R	**105**	**12**	**4**	
AUH-379	R	S	S	S	S	R	S	S	R	**105**	**15**	**52**	
AUH-384	R	S	S	S	S	R	S	S	R	**105**	**15**	**52**	

baumannii, which is frequently detected and clinically important. Strains called "*A. baumannii* international clones" show a tendency toward multidrug resistance and are thought to cause outbreaks[13]. Three strains examined in this study, AUH-352, -359, and -363, were found to be international clones, and they were all presumed to be international clone II, with a POT-1 value of 122[9, 14]. As cases of nosocomial infections have been reported in Japan, it is important to pay close attention to international clones[12]. Although strain AUH-361 could not be identified using the POT method, 16S rRNA gene sequence analysis revealed that it was *A. dispersus*, previously detected in both humans and soil[15]. Unlike other strains, AUH-361 was weakly resistant to PIPC but susceptible to T/P, suggesting that it produces a type of β-lactamase.

Dynamic analysis of the discriminated strains showed that the six strains of *A. baumannii* detected between January and December 2019 could be divided into two clonal groups based on their POT values. However, the nine strains detected between January and December 2020 comprised six groups, indicating that the numbers and types of detected strains increased and diversified over time in the area or medical facility. In Japan, measures must be taken against an outbreak when three or more cases of infectious diseases caused by the same bacterial species are newly identified in the same ward within 4 weeks from the discovery of the first case, or when three or more cases of infectious diseases that are thought to be caused by the same strain are identified within the same medical institution[16]. Although in the present study we did not find cases that met these criteria, caution is needed as the number of detected cases is increasing.

Five strains, AUH-353, -354, -360, -372, and -374, which had POT value of 105-12-20, were clonal. However, unlike the other strains, AUH-360 and -372 acquired DRPM resistance, making them resistant to two drugs (DRPM and LVFX). Similarly, strains AUH-352, -359, and -363 were clonal. Nonetheless, only AUH-363 was resistant to DRPM, whereas only AUH-359 was resistant to CAZ. We plan to analyze drug resistance-related factors in these clonal strains to characterize the different drug resistance patterns in greater detail.

V. Conclusion

Although none of the evaluated drug-resistant *Acinetobacter* spp. strains detected in a northern Osaka Prefecture medical facility in 2019 and 2020 were MDRA, seven were resistant to two drugs (DRPM and LVFX). In addition, we found strains with advanced drug resistance, even within a group classified as clonal based on strain identification, suggesting that resistance trends should be closely monitored.

Conflict of Interest (COI)

The authors have no COI to disclose regarding this study.

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