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Short report

Characterization of *Acinetobacter* clinical isolates

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Abstract

Members of the genus *Acinetobacter* are typical opportunistic bacterial pathogens that can survive for a long term even on inanimate surfaces. *Acinetobacter* species have natural and acquired antibiotic resistance mechanisms that provide resistance against a broad range of antimicrobial agents. Between April 2010 and March 2011, 6 clinical *Acinetobacter* sp. strains were isolated from expectoration or aspiration sputum samples in a local medical treatment-type hospital in Osaka prefecture. The antibiotic susceptibility breakpoint test showed that all the 6 isolates were ciprofloxacin-resistant. Strain AHU-70, which was identified as *A. baumannii* by 16S rRNA sequencing and polymerase chain reaction detection of the $bla_{\text{OXA-51-like}}$ gene, showed high levels of resistance to ciprofloxacin by the minimum inhibitory concentration (MIC) test. Preliminary research in Japan, based on nationwide susceptibility surveillance of ciprofloxacin against *A. baumannii* isolates showed that approximately 90% of the isolates were ciprofloxacin-susceptible. Given these results, further strain level identification of isolates is required to determine whether resistance to ciprofloxacin is an overall trait of these bacteria in the sampled local area or is restricted to a specific strain within particular hospitals.

Key words: *Acinetobacter*, susceptibility, ciprofloxacin

INTRODUCTION

Acinetobacter species constitute prevalent opportunistic human pathogens that cause acute pneumonia, meningitis, and septicemia in immunocompromised patients (Humphreys *et al.*, 1996). They are widely distributed in nature and in hospital environments such as intensive care units, due to their ability for long-term survival even on inanimate surfaces (Kappstein *et al.*, 2000). *A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii, A. lwoffii, A. parvus, A. radioresistens, A. schindleri, A. ursingii, A.* genomic species 3, 13TU, 10, and 11 have been isolated from human specimens (Dortet *et al.*, 2006). Particularly, *A. baumannii* is most frequently isolated from humans and also appears to be the *Acinetobacter* species with the greatest clinical importance. Identification of *Acinetobacter* species on the bases of abbreviated phenotypic tests is difficult, and genetic methods such as standard DNA-DNA hybridization (Bouvet and Grimont, 1986) and sequencing of the 16S rRNA gene (Ibrahim*et al.*, 1997) or the 16-23S rRNA intergenic spacer region (Lee *et al.*, 2011) have been proposed. It is also known that *Acinetobacter* species have natural and acquired antibiotic resistance mechanisms that provide increasing resistance against a broad range of antimicrobial agents (Lee *et al.*, 2011). In Japan, *Acinetobacter* species for which minimum inhibitory concentrations (MICs) of imipenem (IMP), amikacin (AMK), and ciprofloxacin (CPFX) are $\geqq 16\,\mu g/mL$, $\geqq 32\,\mu g/mL$, and $\geqq 4\,\mu g/mL$, respectively, are defined as multidrug-resistant *Acinetobacter* species (MDRA), according to the Infectious Diseases Control Law in correspondence to the increased hospital infection by MDRA (Yuji *et al.*, 2011).

MATERIALS AND METHODS

Bacterial isolates and growth conditions

Between April 2010 and March 2011, clinical *Acinetobacter* sp. strains were isolated from expectoration or aspiration sputum samples of patients with different infections in a medical treatment-type hospital (approximately 1,000 beds) in Osaka prefecture. Genus classification of the strains was performed using the MicroScan WalkAway Plus (Siemens, Germany) fully automated instrument. *A. baumannii* ATCC17978 is a standard strain for which the complete genomic sequence is available (Michael *et al.*, 2007); this strain was isolated from a 4-month-old infant with meningitis. All the strains were cultured at 37℃ in Mueller-Hinton (MH; Oxoid, USA) medium supplemented with antibiotics when appropriate.

DNA techniques

DNA purification and other DNA manipulations were conducted as previously described (Nakada and Itoh, 2002; Sambrook *et al.*, 2001). A polymerase chain reaction (PCR) was performed with KOD-plus DNA polymerase (Toyobo Biochemicals, Japan) in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, USA) under the reaction conditions recommended by the supplier. Fragment size of the PCR products was determined by 1.5% agarose gel electrophoresis.

Nucleotide sequencing of the 16S rRNA gene

The 16S rRNA gene of the AUH-70 strain was amplified by PCR with a universal primer pair of UFPL and URPL (Lipuma *et al.*, 1999; Table 1). PCR conditions were as follows: 94℃ for 3 min; 30 cycles of 94℃ for 1 min, 55℃ for 1 min, and 68℃ for 1.5 min; and a final extension at 68℃ for 4 min. The resultant approximately 1.5-kbp fragment was purified using a PCR Purification Kit (WAKO, Japan), and its nucleotide sequence was determined using the ABI3130 DNA sequencer and a Big-Dye Terminator Sequencing Kit (Applied Biosystems, USA). Sequence assembly was performed using ATGC version 5.0 (Genetyx Corporation, Japan), and the resultant sequence was compared with those in the NCBI database by using a BLAST program.

Detection of the $bla_{\text{OXA-51-like}}$ gene

The $bla_{\text{OXA-51-like}}$ gene, which appears to be intrinsic to *A. baumannii* (Woodford *et al.*, 2006), was detected by PCR by using the primer pair of OXA-51-likeF and OXA-51-likeR (Table 1). PCR conditions were as follows: 94℃ for 3 min; 28 cycles of 94℃ for 1 min, 60℃ for 1 min, and 68℃ for 1 min; and a final extension at 68℃ for 4 min.

Antibiotic susceptibility test

Antimicrobial breakpoint tests were conducted by FALCO biosystems Ltd. by using the MicroScan Walk Away Plus (Siemens, Germany) fully automated instrument. Isolates were determined to be susceptible (S), intermediate (I), or resistant (R) according to the antibiotic breakpoint guidelines of the Clinical and Laboratory Standards Institute (CLSI) for *Acinetobacter* sp. (CLSI, 2006).

The MICs for IMP, AMK, and CPFX, which are used to define MDRA in Japan, were also determined by the standard broth dilution method according to the guidelines of the CLSI. Overnight cultures of each *Acinetobacter* sp. strain were diluted in saline to an optical density of 0.1 at 600 nm of (approximately 1×10^8 CFU/mL), and a portion of the adjusted cell suspension ($\sim 10^5$cells) was inoculated on the MH medium containing antibiotics as indicated. The cell cultures were then incubated overnight at 37℃.

RESULTS

Isolated strains

Within one year, 6 patients developed *Acinetobacter* sp. infection, and clinical isolates, including AUH-70 strain, were obtained from expectoration or aspiration sputum samples (Table 2). The patients included those in old-age chronic stage wards (A, B) and specific disease wards (C).

Identification of the AUH-70 strain

The 16S rRNA genomic region containing approximately 1500 nucleotides was amplified (Fig. 1), and 1406 bp sequence data were obtained by direct sequencing. Alignment and comparison

Table 1 PCR primers and their target genes and amplicon sizes

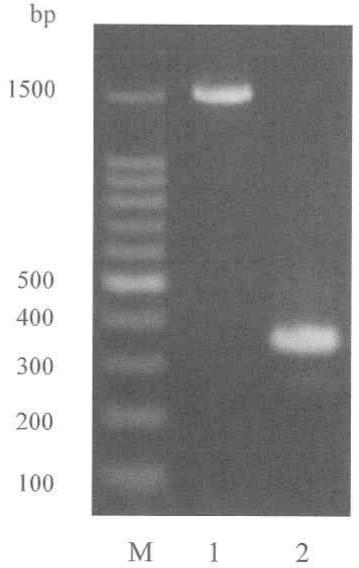
Primer	Oligonucleotide sequence (5'-3')	Target	Amplicon size (bp)
UFPL	AGTTTGATCCTGGCTCAG	16SrRNA gene	1492*
URPL	GGTTACCTTGTTACGACTT		
OXA-51-likeF	TAATGCTTTGATCGGCCTTG	$bla_{\text{OXA-51-like}}$	353
OXA-51-likeR	TGGATTGCACTTCATCTTGG		

*Corresponding to positions 9-1500 in the *Escherichia coli* numbering system

Table 2 Specimens and antimicrbial breakpoints of *Acinetobacter* sp. isolates

Isolate code (Strain)	Hospital ward	Specimens	Breakpoint					
			PIPC	CAZ	IPM	MINO	AMK	CPFX
E41	A	aspiration sputum	R	S	S	S	S	R
N21	B	expectoration	S	S	S	S	S	R
C41	C	aspiration sputum	R	S	S	S	S	R
C42	C	aspiration sputum	R	S	S	S	S	R
N22 (AUH-70)	B	aspiration sputum	S	S	S	S	S	R
C43	C	aspiration sputum	S	S	S	S	S	R

A and B: old-age chronic stage wards, C: specific disease wards.
PIPC: piperacillin, CAZ: ceftazidime, IPM: imipenem, MINO: minocycline, AMK: amikacin, CPFX: ciprofloxacin.



Lane 1: 16S rRNA gene, lane 2: $bla_{\text{OXA-51-like}}$ gene, M: 100-bp DNA marker

Fig. 1 PCR results of amplified fragments of the 16S rRNA gene and $bla_{\text{OXA-51-like}}$ gene from the AUH-70 strain

of this region with the *A. baumannii* ATCC17978 16S rRNA gene which is positioned from nt 53 to nt 1458 (Michael *et al.*, 2007), indicated complete homology. Moreover, sequence similarity with the equivalent 16S rRNA gene region of *A. baumannii* UMB001, *A. baumannii* ABNIH1, *A. baumannii* ATCC19606, and *A. baumannii* AYE was greater than 99.9%. Furthermore, PCR detection of the $bla_{\text{OXA-51-like}}$ gene, which appears to be intrinsic to *A. baumannii*, was positive (Fig. 1). These results suggest that the AUH-70 strain was *A. baumannii*.

Antibiotic susceptibility

The antimicrobial breakpoint test showed that all 6 isolates were CPFX-resistant; moreover, of these 6 isolates, 3 were piperacillin-resistant (Table 2). Furthermore, the resultant of MIC test showed that the AUH-70 strain was highly resistant to CPFX (Table 3). On the other hand, IMP and AMK was still effective to the clinically

Table 3 MICs of *A. baumannii* ATCC17978 and the AUH-70 strain

Strain	MICs (μg/mL)		
	Imipenem	Amikacin	Ciprofloxacin
A. baumannii ATCC17978	0.25	0.5	0.125
A. baumannii AUH-70	0.25	0.5	32

isolated AUH-70 strain, as was *A. baumannii* ATCC17978 standard strain.

DISCUSSION

In this study, we examined the antibiotic susceptibility of *Acinetobacter* sp. isolates obtained from a medical treatment-type hospital in Osaka prefecture. Within one year, 6 patients developed *Acinetobacter* sp. infection, and all isolates obtained from these patients showed CPFX resistance. CPFX is a fluoroquinolone-type antibiotic that exerts its antibacterial effects by inhibiting DNA gyrase and topoisomerase Ⅳ enzymes, which are necessary for separating bacterial DNA, thereby inhibiting cell division. Nationwide susceptibility surveillance studies of CPFX against *A. baumannii* isolated from patients between 2001 and 2005 (Yamaguchi *et al*, 2008) and between 2006 and 2010 (http: //www. kansensho. or. jp/mrsa/index. html) showed that approximately 90% of the collected isolates were susceptible to this antibiotic. These results suggest 2 possibilities. One is the rapid evolution of the CPFX-resistant *Acinetobacter* sp. in the sampled local area, and the other is the development of a few CPFX-resistant strains in a particular hospital. In the current study, the number of affected patients in specific wards, and the limited variation in antibiotic susceptibility patterns, seem to support the likelihood of expansion of a few resistant strains in the hospital; however there is no conclusive evidence for this

hypothesis. From the standpoint of nosocomial infection control, a surveillance study involving strain identification by REP-PCR, VNTR, or PFGE (Pourcel *et al*, 2011) is required.

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