

Development of a Novel Immunochromatographic Assay for Rapid Detection of OXA-23 β -lactamase-producing *Acinetobacter baumannii*

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Among the several agents causing carbapenem resistance of *Acinetobacter baumannii*, the most common cause is OXA-23 β -lactamase, which is known to hydrolyze carbapenem. To effectively control dissemination of carbapenem-resistant *Acinetobacter baumannii* (CRAB), development of both rapid and easy-to-use detection methods are required. The aim of this study is to develop a novel immunochromatographic assay (ICA) for rapid detection of OXA-23 β -lactamase. Of the seven monoclonal antibodies (mAbs) screened by ELISA, four mAbs (4G6, 4H6, 6G4, 9A4) exhibited high reactivity. Of these four specific antibodies, the combination of 6G4/4G6 showed the greatest reactivity and this combination of mAbs (6G4/4G6 mAbs) was used to develop the OXA-23 β -lactamase ICA. Of 102 *A. baumannii* isolates tested, the OXA-23 β -lactamase ICA results were consistent with PCR analysis except one false positive and one false negative isolate. The overall sensitivity and specificity were 98.36% and 97.56%, respectively. In conclusion, to the best of our knowledge, we have developed the first specific antibody set to detect OXA-23 β -lactamase using an ICA kit. This novel ICA can be used as a reliable and easy-to-use immunological assay for detection of OXA-23 β -lactamase producing CRAB in clinical laboratories.

Key Words: *Acinetobacter baumannii*, OXA-23 β -lactamase, Immunochromatographic assay, Rapid detection

INTRODUCTION

Acinetobacter baumannii frequently causes various nosocomial infections worldwide, including ventilator-associated pneumonia, bacteremia, and urinary tract infections (Munoz-Price and Weinstein, 2008; Peleg et al., 2008; Villegas and Hartstein, 2003). In particular, nosocomial infections caused by this species often occur in patients hospitalized in intensive care and burn units (Wilks et al., 2006). Recently, the prevalence of multidrug-resistance in *A. baumannii* has

dramatically increased (Lee et al., 2006; Lee et al., 2009; Lee et al., 2009; Sung et al., 2011). Carbapenem-resistant *Acinetobacter* spp. are increasing due to the emergence of carbapenem-hydrolyzing β -lactamases belonging to molecular classes B and D (Walther-Rasmussen and Hoiby, 2006; Lee et al., 2009). Whereas class B carbapenemase, a metallo- β -lactamase (MBL), have been frequently founded in non-*baumannii* members of this genus, an increase in class D carbapenemases has been reported in *A. baumannii* in South Korea (Lee et al., 2009; Lee et al., 2007). It is known that class D carbapenemase in *A. baumannii* and

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Table 1. Oligonucleotide primers used in this study to detect OXA type carbapenemase

Name	Primer sequence (5' → 3')	Target gene	Reference
OXA23-F	GAT CGG ATT GGA GAA CCA GA	<i>bla</i> _{OXA-23} -like	(Woodford et al., 2006)
OXA23-R	ATT TCT GAC CGC ATT TCC AT		
OXA24-F	GGT TAG TTG GCC CCC TTA AA	<i>bla</i> _{OXA-24} -like	(Woodford et al., 2006)
OXA24-R	AGT TGA GCG AAA AGG GGA TT		
OXA51-F	TAA TGC TTT GAT CGG CCT TG	<i>bla</i> _{OXA-51} -like	(Woodford et al., 2006)
OXA51-R	TGG ATT GCA CTT CAT CTT GG		
OXA58-F	AAG TAT TGG GGC TTG TGC TG	<i>bla</i> _{OXA-58} -like	(Woodford et al., 2006)
OXA58-R	CCC CTC TGC GCT CTA CAT AC		

class B carbapenemase in non-*baumannii* are principal agents which cause the resistance to carbapenem in these strains (Lee et al., 2009). The OXA-type class D carbapenemases of *A. baumannii* can be divided into the following subgroups: OXA-23, OXA-24, OXA-51 and OXA-58. Of these, OXA-23 and OXA-51 are the most common and frequently occur simultaneously within a single isolate. While OXA-51 is an intrinsic enzyme, OXA-23 is an acquired enzyme and it frequently leads to *A. baumannii* outbreaks in intensive care units around the world (Jeong et al., 2006; Lee et al., 2009; Zong et al., 2008).

In this study, we designed an immunochromatographic assay using novel monoclonal antibodies (mAbs) for OXA-23 β -lactamase and evaluated the performance of the rapid kit using carbapenem-resistant *Acinetobacter baumannii*.

MATERIALS AND METHODS

Bacterial isolates

A total of 102 non-duplicated *A. baumannii* strains resistant to carbapenem (including imipenem or meropenem) were collected from hospital in South Korea. The identification of all strains was initially carried out by biochemical tests using the Vitek II system (bioMérieux, Hazelwood, MO, USA), and subsequently confirmed by the detection of *bla*_{OXA-51} gene (Turton et al., 2006). The susceptibility of the strains to carbapenem or the other antibiotics was screened by disk diffusion method or Vitek II susceptibility card (CLSI, 2012). The confirmative test of antibiotics including imipenem was performed by E-test (bioMérieux, Inc., Durham,

NC, USA).

Antimicrobial susceptibility testing and screening for carbapenemase production

The minimal inhibitory concentrations (MICs) of imipenem were determined for the bacterial isolates by the Clinical and Laboratory Standards Institute (CLSI) E-test or broth dilution method (CLSI, 2012). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains for antimicrobial susceptibility testing. The modified IPM disk Hodge test was used to screen for carbapenemase and IPM-EDTA double disk synergy test was used to screen for MBL (Lee et al., 2003).

Amplification and sequencing of antimicrobial resistance genes

The genes encoding OXA-type carbapenemases were detected through a multiplex PCR using the genomic DNAs as templates and the reaction conditions were described by Woodford et al. (Woodford et al., 2006). GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA, USA) was used for the amplification and the primers and PCR reaction conditions were described in Table 1. The amplified DNA products were purified according to the manufacturer's instruction (Promega, Madison, WI, USA) and sequenced using an ABI3130XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were compared with those in the GenBank nucleotide database (www.ncbi.nlm.nih.gov/blast/). In addition, previously reported PCR primers were used to determine the presence

of *bla*_{IMP-1}, *bla*_{VIM-1}, or *bla*_{VIM-2} (Yum et al., 2002), which is class B carbapenemase (metallo-β-lactamase).

Expression and purification of recombinant OXA-23 β-lactamase

The genes encoding the OXA-23 β-lactamase originated from *A. baumannii* was synthesized (BioBasic Inc., Toronto, Canada). A synthesized gene of 822 bp, corresponding to OXA-23 β-lactamase, was directly ligated into the pET101/D-TOPO cloning and expression vector (Invitrogen, CA, USA) by the heat-shock method (Bergmans et al., 1981). The ligation products were transformed into competent *Escherichia coli* BL21 StarTM (DE3) cells (Invitrogen), and

the transformants were selected on LB agar containing 50 μg/mL ampicillin. Expressed histidine-tagged proteins were purified by immobilized metal ion affinity chromatography (Hochuli et al., 1988). The purified fraction from Ni-resin affinity chromatography was confirmed by 12% SDS PAGE, and a band was obtained corresponding to about 30 kDa.

Preparation of monoclonal or polyclonal antibodies specific for OXA-23 β-lactamase

Anti-OXA-23 mAbs were prepared as described previously (Stähli et al., 1980). Purified His-tagged recombinant OXA-23 β-lactamase was used for immunization and screening of hybridomas by indirect enzyme-linked immunosorbent

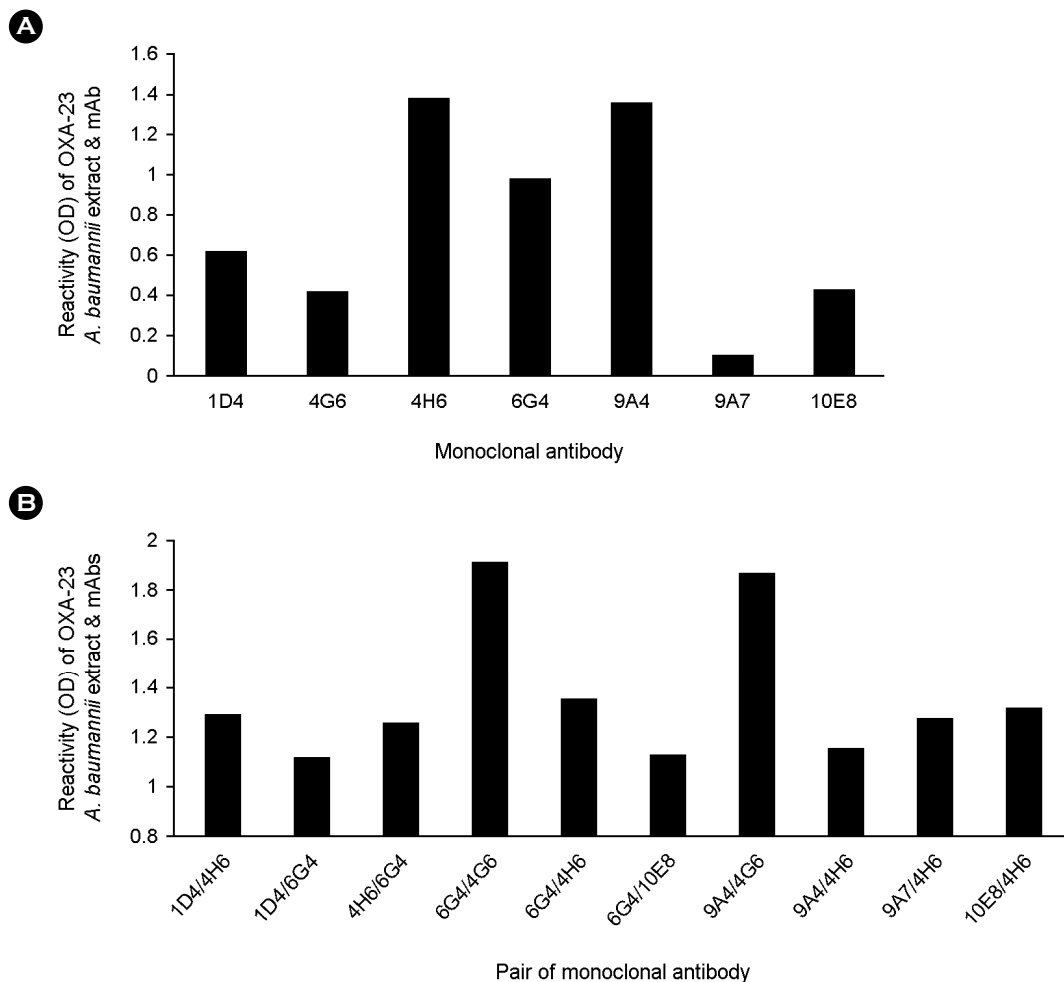


Fig. 1. Selection of monoclonal antibody and pair to detect OXA-23 β-lactamase by indirect and sandwich ELISA. Seven mAbs selected by ELISA showed reactivity (OD) of OXA-23 producing *A. baumannii* extract and mAbs (A). A pair of mA 6G4/4G6 revealed the greatest reactivity (B).

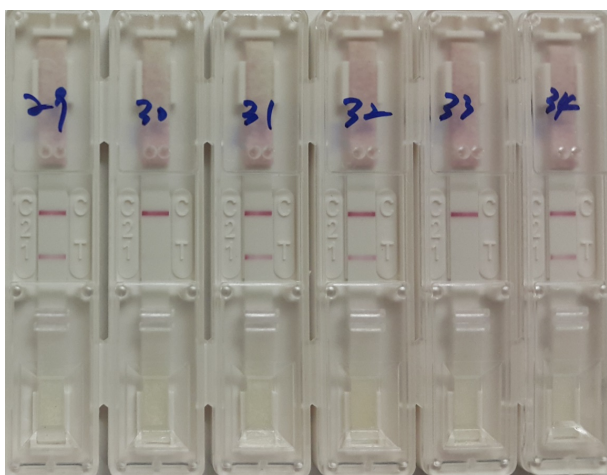


Fig. 2. The results of immunochromatographic assay to detect OXA-23 β -lactamase in carbapenem-resistant *Acinetobacter baumannii*. Six *A. baumannii* isolates were tested using the developed ICA kit. Isolate #29, #31, #32, and #34 (bla_{OXA-23} gene positive) show two distinct bands on the control and test line (positive result) but isolate #30 and isolate #33 isolates (bla_{OXA-23} negative) only show a band in control line (negative result).

assay (ELISA). Among the anti-OXA-23 mAbs selected by ELISA, various mAbs combinations were evaluated for detection of OXA-23 β -lactamase. The animal experiments were approved by the Ethical Committee for Animal Experiments at the Research Institute of Dinona Co. (approval number: DN13-RD-CH05).

Determination of mAbs to detect OXA-23 β -lactamase and preparation of the ICA kit

The best combination of mAbs acting as antibody for OXA-23 β -lactamase was determined by indirect ELISA and sandwich ELISA. To improve the sensitivity of ICA, rabbit polyclonal antibody was induced by recombinant OXA-23 β -lactamase and then this polyclonal antibody was used as the capture antibody. To prepare the test and control lines of ICA, 1.0 mg of rabbit poly-antibody specific for OXA-23 β -lactamase (as the capture antibody) and 0.3 mg of anti-mouse IgG (Dinona, Iksan, South Korea) per test were coated onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) at 30 mm and 35 mm distal from the sample application area, respectively. Gold colloid conjugated with anti-OXA-23 β -lactamase monoclonal IgG (as

the detector antibody) was sprayed onto a conjugated pad (Millipore) placed on an adjacent sample pad and lyophilized overnight.

Assesment of the immunochromatographic assay using clinical isolates

To evaluate the performance of ICA for OXA-23 β -lactamase, the sensitivity and specificity were determined using carbapenem resistant *A. baumannii* with PCR for bla_{OXA-23} in two laboratories. To evaluate the cross reactivity with other bacteria, the carbapenem resistant *Pseudomonas species*, *Achromobacter xylosoxidans* and the other gram negative or positive bacteria were tested by ICA for OXA-23 (Table 3).

RESULTS

Selection of anti-OXA-23 β -lactamase mAbs and preparation of ICA kit

The seven mAbs for OXA-23 β -lactamase were selected by indirect ELISA. To determine the specific antibody combination for OXA-23 β -lactamase, various mAb pairs from the seven mAbs were evaluated with the extract of OXA-23 *A. baumannii*. Four antibodies such as 4G6, 4H6, 6G4, 9A4 revealed great reactivity for OXA-23 β -lactamase. Among four mAbs (4G6, 4H6, 6G4, 9A4), 6G4/4G6 pair showed the greatest reactivity (Fig. 1). In addition to, a rabbit anti-OXA-23 polyclonal antibody was induced by recombinant OXA-23 β -lactamase and then used for capture antibody to improve the sensitivity of ICA. To develop ICA kit, rabbit anti-OXA-23 polyclonal antibody was immobilized on the nitrocellulose membrane for capture and four mAbs (4G6, 4H6, 6G4, 9A4) were labeled with colloidal-gold particle as the detector of OXA-23. Of the four combinations, rabbit polyclonal antibody capture plus 6G4 detector showed the greatest reactivity against the extract of OXA-23 producing *A. baumannii*; therefore, this combination was used to prepare the OXA-23 β -lactamase ICA (Fig. 2).

Assesment of the assay using clinical isolates

Of 102 *A. baumannii*, 61 were positive for bla_{OXA-23} gene and 41 isolates were negative for bla_{OXA-23} gene by PCR

Table 2. Diagnostic performance of the immunochromatographic assay for OXA-23 β -lactamase in clinical isolates cultured from MacConkey agar compared with PCR detection of OXA-23 β -lactamase producing *Acinetobacter baumannii*

Microorganisms (n)	PCR for <i>bla</i> _{OXA-23}	ICA for OXA-23 β -lactamase			
		A-Lab		B-Lab	
		P	N	P	N
<i>CR-Acinetobacter baumannii</i> (102)	P	60	1	59	2
	N*	1	40	0	41
	Sensitivity (%)	98.36		96.72	
	Specificity (%)	97.56		100	
<i>CR-Pseudomonas</i> spp. (33) [†]	N	0	33	0	33
<i>CR-Achromobacter xylosoxidans</i> (9) [‡]	N	0	9	0	9
Total (144)	P	60	1	59	2
	N	1	82	0	83
	Sensitivity (%)	98.36		96.72	
	Specificity (%)	98.80		100	

Abbreviations: CR, carbapenem resistant; ICA, immunochromatographic assay; P, positive; N, negative

*Among 41 *CR-A. baumannii* without *bla*_{OXA-23} included 19 IMP-1 and 13 VIM-2 type metallo- β -lactamase (MBL) producing isolates.

[†]*CR-Pseudomonas* (*P. aeruginosa*, *P. fluorescens* and *P. putida*) spp. included 32 VIM-2 type MBL producing isolates.

[‡]All of *CR-A. xylosoxidans* was VIM-2 type MBL producing isolates.

analysis. Sixty isolates among 61 isolates with *bla*_{OXA-23} gene were positive by ICA. Forty isolates among 41 isolates without *bla*_{OXA-23} gene were negative by ICA in A-laboratory. In B-laboratory, 100/102 was consistent with PCR results except 2 false negative. The overall sensitivity and specificity were 98.36% and 97.56% in A-laboratory, 96.72% and 100% in B-laboratory, respectively (Table 2). To evaluate cross reactivity with other bacteria, the *CR-P. aeruginosa*, *A. xylosoxidans* and the other gram negative or positive bacteria were tested by ICA for OXA 23. The ICA kit revealed the negative result from all non-*A. baumannii* (Table 3 & 4).

DISCUSSION

Although rapid detection methods such as real-time PCR assay are used routinely to control the spread of the infection by multidrug-resistant (MDR) microorganism, including CRAB, MRSA, VRE in many clinical laboratories, conventional bacterial cultures are used to isolate MDR microorganism from clinical specimens and to confirm the presence of MDR in specimens containing a mixed bacterial population. Moreover, this approach allows antimicrobial susceptibility testing for selection of an appropriate anti-

Table 3. Results of immunochromatographic assay for OXA-23 β -lactamase in gram positive cocci and yeasts

Species	A-Lab	B-Lab
<i>Staphylococcus aureus</i> ATCC 29213	Negative	Negative
<i>Staphylococcus aureus</i>	Negative	Negative
<i>Streptococcus pneumoniae</i>	Negative	Negative
<i>Streptococcus agalactiae</i>	Negative	Negative
<i>Streptococcus viridans</i> group	Negative	Negative
<i>Enterococcus faecalis</i> ATCC 29212	Negative	Negative
<i>Enterococcus faecalis</i>	Negative	Negative
<i>Enterococcus faecium</i>	Negative	Negative
<i>Staphylococcus epidermidis</i>	Negative	Negative
<i>Staphylococcus hominis</i>	Negative	Negative
<i>Candida albicans</i>	Negative	Negative
<i>Candida parapsilosis</i>	Negative	Negative
<i>Candida tropicalis</i>	Negative	Negative

microbial treatment. After CRAB have been cultured and identified, molecular methods are necessary to determine their genotype (Class B or D: OXA genotypes); such methods are labor-intensive and costly yet indispensable, and their use other than in epidemiological investigations is controversial.

Table 4. Results of immunochromatographic assay for OXA-23 β -lactamase in gram negative bacilli

Species	A-Lab	B-Lab
<i>Escherichia coli</i> ATCC 25922	Negative	Negative
<i>Pseudomonas aeruginosa</i> ATCC 27853	Negative	Negative
<i>Klebsiella oxytoca</i> ATCC 700324	Negative	Negative
<i>Escherichia coli</i>	Negative	Negative
<i>Klebsiella pneumoniae</i>	Negative	Negative
<i>Klebsiella oxytoca</i>	Negative	Negative
<i>Enterobacter aerogenes</i>	Negative	Negative
<i>Enterobacter cloacae</i>	Negative	Negative
<i>Pseudomonas aeruginosa</i>	Negative	Negative
<i>Stenotrophomonas maltophilia</i>	Negative	Negative
<i>Burkholderia cepacia</i>	Negative	Negative
<i>Acinetobacter baumannii</i> (IMP-S)	Negative	Negative
<i>Pseudomonas putida</i>	Negative	Negative

Abbreviation; IMP-S, imipenem susceptible

The immunological assay may be useful for confirming the genotype of CRAB in this situation, which is encountered daily in microbiological laboratories worldwide. Moreover, this OXA-type carbapenemase ICA can incorporate multiple test lines and will facilitate control of nosocomial infection by OXA-23 CRAB together with ICAs for various OXA-type carbapenemase. In this study, we developed a specific mAbs for rapid detection of OXA-23 β -lactamase. Seven mAbs to OXA-23 β -lactamase were selected, one pair (6G4/4G6) mAb showed the greatest reactivity. However, the ICA using this mAb showed a weak signal in the kit. To improve the sensitivity of ICA kit, a rabbit polyclonal antibody by recombinant OXA-23 β -lactamase was developed and was used as a capture antibody of ICA with detector antibody (6G4 mAb). The kit produced a distinct signal and the rabbit polyclonal antibody was used capture antibody of ICA (Fig. 2).

Of 102 *A. baumannii* isolates, 61 isolates were positive for *bla*_{OXA-23} gene and 41 isolates were negative for *bla*_{OXA-23} gene by PCR analysis. Sixty isolates among 61 isolates with *bla*_{OXA-23} gene were positive by ICA. Forty isolates among 41 isolates without *bla*_{OXA-23} gene were negative by ICA in A-laboratory (Table 2). In addition, except for two false

negative results, 100 of 102 isolates were consistent with PCR results in B-laboratory. The overall sensitivity and specificity were 98.36% and 97.56% in A-laboratory, 96.72% and 100% in B-laboratory, respectively (Table 2). To evaluate cross reactivity with other bacteria, the carbapenem resistant *P. aeruginosa*, *A. xylosoxidans* and the other gram negative or positive bacteria were tested by ICA for OXA-23. The ICA kit revealed a negative result from all non-*A. baumannii* (Table 3).

The limitation of this study is that the performance of the developed ICA kit was evaluated using the colony grown on agar plates rather than direct clinical samples for overcoming the low sensitivity of this kit. Therefore, the newly developed ICA kit requires the more consuming time than a PCR method to directly detect OXA-23 β -lactamase from clinical samples. In addition, a considerable quantity of bacterial colony is required to test of ICA kit. Furthermore, the evaluation for detection limit and conditions to improve the sensitivity, such as enrichment incubation with carbapenem, which induce the OXA-23 β -lactamase, are required.

However, because rapid and easy detection of CRAB is the primary object of ICA and most laboratory usually isolated bacteria on agar plates, ICA is sufficient to detect OXA-23 producing *A. baumannii* in clinical laboratories. The cost of using this kit is estimated to be only 10% of the cost of performing real-time PCR, which is the current method of detecting the OXA-23 β -lactamase gene in South Korea, making it affordable for most clinical laboratories. In addition, whereas many molecular methods require batch operations by trained personnel, this rapid kit assay can be used with few samples and by non-specialist workers (Shin et al., 2010; Ji et al., 2014).

In conclusion, the novel ICA assay is easy-to-use, and rapid immunologic method for the detection of OXA-23 β -lactamase-producing *A. baumannii* from conventionally cultured bacterial colonies. To our knowledge, this is the first report of an immunological detection assay for OXA-23 β -lactamase. Additional development of rapid immunological assays for OXA-24, OXA-48 and OXA-51 will facilitate the control of CRAB infection and investigation of its epidemiology.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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