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Simultaneous Identification of Multiple β-Lactamases in *Acinetobacter baumannii* in Relation to Carbapenem and Ceftazidime Resistance, Using Liquid Chromatography-Tandem Mass Spectrometry

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Shotgun proteomics using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied to detect β-lactamases in clinical *Acinetobacter baumannii* isolates. The correlation of the detection of β-lactamase proteins (rather than PCR detection of the corresponding genes) with the resistance phenotypes demonstrated an added value for LC-MS/MS in antimicrobial susceptibility testing.

Rapid detection and identification of β-lactamase-related resistance are complicated by the increasing variety of β-lactamases (with differences in substrate specificity), as well as the lack of information on the expression of particular β-lactamases for DNA-based detection. Recently, methods based on mass spectrometry, either to identify resistance-conferring proteins (1, 2) or to assay their activity (3, 4), have been developed. In this study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to detect and to identify oxacillinases and other β-lactamases in clinical isolates of the nosocomial pathogen *Acinetobacter baumannii*, in order to evaluate its potential as a rapid and generic method for the detection of β-lactamase-related resistance. Emerging multidrug resistance in *A. baumannii*, resulting from innate resistance to multiple classes of antimicrobials along with a large capacity for acquiring resistance, is an increasing concern in hospitals; the recent rapid development and spread of resistance against carbapenems are of particular concern (5, 6). Resistance is usually caused by the activity of intrinsic or acquired carbapenem-hydrolyzing class D β-lactamases, also known as oxacillinases. Although oxacillinases are considered weak carbapenem hydrolyzers, strains become resistant when the genes are strongly expressed (7). Similarly, *A. baumannii* resistance against ceftazidime resulting from overexpression of *Acinetobacter*-derived cephalosporinase (ADC), a chromosomally encoded, AmpC-type β-lactamase in *A. baumannii*, has been reported (8). Elevated expression of β-lactamase genes in *A. baumannii* is often associated with the presence of an insertion element (IS) (in particular, IS*Aba1*) upstream of the β-lactamase gene, providing strong promoter elements (9). The LC-MS/MS results in this study were compared with the results of susceptibility tests, PCR tests for the presence of different β-lactamase genes and insertion elements, and sequencing of detected β-lactamase genes.

A total of 29 *A. baumannii* isolates from blood and wound infections, collected at the San Antonio Military Medical Center (San Antonio, TX) between 2006 and 2008, were studied. Pulsed-field gel electrophoresis (PFGE) analysis revealed that these clustered into 15 different PFGE types, with a maximum of 5 isolates belonging to a PFGE type (see Fig. S1 in the supplemental material) (10, 11). Resistance to the carbapenems imipenem and meropenem and the third-generation cephalosporin ceftazidime was determined by broth microdilution testing (12) (Table 1). Fifteen isolates were resistant to both carbapenems, 11 were susceptible, and three showed intermediate levels of resistance to either imipenem or meropenem. Twenty-five isolates were resistant to ceftazidime, three were susceptible, and one showed an intermediate level of resistance. PCR assays using the primers described in Table S1 in the supplemental material were performed to assess the presence of 15 different β-lactamase genes (Table 1), followed by sequencing of the complete genes, except $bla_{\text{ADC-like}}$, and also 1 kb upstream of the gene when an insertion element was detected by using IS*Aba1*-specific primers or by applying inverse PCR. All isolates scored positive for the intrinsic $bla_{\text{OXA-51-like}}$ and $bla_{\text{ADC-like}}$ genes. Eleven isolates (38%) scored positive for $bla_{\text{OXA-23-like}}$ and four isolates (14%) for $bla_{\text{OXA-40-like}}$, which upon sequencing appeared to encode OXA-23 and OXA-40, respectively. These isolates were all resistant to both tested carbapenems, whereas all other isolates were susceptible or showed intermediate levels of resistance (Table 1). A $bla_{\text{CMY-2-like}}$ gene (encoding plasmid-mediated AmpC-type cephalosporinase) was detected in one isolate and encoded CMY-30, a close homologue of CMY-2 with comparable activity (13). To our knowledge, the presence of $bla_{\text{CMY-2-like}}$ in *A. baumannii* has not been reported previously. A $bla_{\text{PER-1-like}}$ gene was detected in isolates 10, 20, and 22, which upon sequencing appeared to encode PER-1 with the insertion element IS*Pa12* upstream of the gene (18). $bla_{\text{GES-1-like}}$ was detected in isolate 29 and encoded the GES-11 variant, which has

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TABLE 1 MICs of imipenem, meropenem, and ceftazidime for 29 clinical *A. baumannii* isolates, identification of β-lactamase genes and upstream IS elements by PCR, and LC-MS/MS detection of β-lactamase proteins

Isolate no.	MIC (mg/liter)[a]			*bla*$_{OXA-51-like}$/OXA-51-like			*bla*$_{OXA-23-like}$/OXA-23-like			*bla*$_{OXA-40-like}$/OXA-40-like			*bla*$_{ADC-like}$/ADC-like			Other β-lactamases			PFGE type
	IPM	MEM	CAZ	PCR	IS	LC-MS/MS	PCR	IS	LC-MS/MS	PCR	IS	LC-MS/MS	PCR	IS	LC-MS/MS	PCR	IS	LC-MS/MS	
1	4	8	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				10
2	4	4	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				10
3	2	1	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				3
4	4	8	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				7
5	8	4	64	+	−	−	−		−	−		−	+	−	−	*bla*$_{CMY-2-like}$	−	CMY-2-like	6
6	4	4	64	+	IS*Aba1*	+	−		−	−		−	+	IS*Aba1*	+				7
7	4	4	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				13
8	4	2	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				12
9	4	4	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				10
10	4	1	64	+	−	−	−		−	−		−	+	−	−	*bla*$_{PER-1-like}$	IS*Pa12*[b]	PER-1-like	8
11	4	4	8	+	−	−	−		−	−		−	+	−	−				14
12	2	0,5	8	+	−	−	−		−	−		−	+	−	−				2
13	4	4	8	+	−	−	−		−	−		−	+	−	−				9
14	32	16	64	+	−	−	−		−	+	−	+	+	IS*Aba1*	+				1
15	32	16	64	+	−	−	−		−	+	−	+	+	IS*Aba1*	+				1
16	32	16	64	+	−	−	−		−	+	−	+	+	IS*Aba1*	+				1
17	32	16	64	+	−	−	−		−	+	−	+	+	IS*Aba1*	+				1
18	32	16	16	+	IS*Aba16*	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				12
19	32	16	32	+	IS*Aba16*	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				12
20	32	16	64	+	−	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+	*bla*$_{PER-1-like}$	IS*Pa12*	PER-1-like	15
21	32	16	32	+	−	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				12
22	32	16	64	+	−	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+	*bla*$_{PER-1-like}$	IS*Pa12*	PER-1-like	15
23	32	16	32	+	IS*Aba16*	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				12
24	32	16	64	+	−	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				5
25	32	16	64	+	−	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				5
26	32	16	64	+	−	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				5
27	4	4	64	+	−	−	−		−	−		−	+	IS*Aba1*	+				7
28	32	16	32	+	IS*Aba16*	−	+	IS*Aba1*	+	−		−	+	IS*Aba1*	+				12
29	32	16	64	+	−	−	+	IS*Aba1*	+	−		−	+	−	−	*bla*$_{GES-1-like}$	−	GES-1-like	4

[a] CLSI breakpoints for imipenem and meropenem were as follows: susceptible, ≤4 mg/liter; intermediate, 8 mg/liter; resistant, ≥16 mg/liter. Breakpoints for ceftazidime were as follows: susceptible, ≤8 mg/liter; intermediate, 16 mg/liter; resistant, ≥32 mg/liter (12). IPM, imipenem; MEM, meropenem; CAZ, ceftazidime.

[b] Isolate 10 contained an additional 44-bp fragment between the left inverted repeat of IS*Pa12* and the start codon of *bla*$_{PER-1}$, compared to isolates 20 and 22 (18).

enhanced activity toward ceftazidime (14). The insertion element IS*Aba1* was detected upstream of $bla_{\text{OXA-51-like}}$ in isolate 6, upstream of all detected $bla_{\text{OXA-23-like}}$ genes, and upstream of $bla_{\text{ADC-like}}$ in 23 isolates (Table 1). In four isolates, a transposase gene (transposase C) previously described as part of the insertion element IS*Aba16* (15) was detected directly upstream of $bla_{\text{OXA-51-like}}$, which encoded OXA-64 in those isolates.

Shotgun proteomics analysis of all 29 isolates was performed in duplicate in separate experiments. Isolates were grown overnight on tryptic soy agar (TSA) plates at 37°C. Approximately 10^9 cells were resuspended in 100 μl of 100 mM ammonium bicarbonate and incubated at 100°C for 10 min. Dithiothreitol (DTT) and trypsin were added to final concentrations of 5 mM and 10 μg/ml, respectively, and samples were incubated for 1 h at 37°C. Trypsin digestion was stopped by adding formic acid to a final concentration of 0.1%. Large particles were removed by centrifugation (20,000 × *g* for 1 min), and supernatants were filtered through a Microcon centrifugal filter device with a cutoff size of 30 kDa (Merck Millipore). The digests were analyzed with LC-MS/MS using a nano-Advance liquid chromatography system (Bruker Daltonics GmbH, Bremen, Germany) coupled to a quadrupole time of flight (Q-TOF) mass spectrometer (maXis impact; Bruker), as described previously (16). Data were analyzed using the Mascot search algorithm (Mascot 2.2.04; Matrix Science, London, United Kingdom), and proteins were considered identified when the protein score was 50 or higher and when at least two peptides were identified.

For all isolates that scored positive for $bla_{\text{OXA-23-like}}$ or $bla_{\text{OXA-40-like}}$ in the PCR screening and were resistant to both tested carbapenems, OXA-23-like or OXA-40-like was identified, with identified peptides covering 26 to 73% (OXA-23-like) or 8 to 25% (OXA-40-like) of the amino acid sequences of the complete proteins (Table 1; also see Table S1 in the supplemental material). OXA-51-like and ADC-like proteins were detected only in the isolates in which IS*Aba1* was located upstream of the corresponding genes (isolate 6, OXA-51-like; 23 isolates, ADC-like) (Table 1), suggesting that IS*Aba1* enhances the expression of these chromosomally located genes to levels that are well detectable with the method described. The overexpression of $bla_{\text{OXA-51-like}}$ in isolate 6, encoding OXA-71 (see Fig. S1 in the supplemental material), did not result in resistance to the tested carbapenems, indicating that OXA-71 has little activity against carbapenems. The isolates that overexpressed $bla_{\text{ADC-like}}$ were all resistant to ceftazidime, which is in agreement with previous work (8, 17). In the three ceftazidime-resistant isolates in which no ADC-like protein was detected, other β-lactamases with known cephalosporinase activity were identified, i.e., CMY-2-like in isolate 5, PER-1-like in isolate 10, and GES-1-like in isolate 30 (Table 1), which is in accordance with the detection of $bla_{\text{CMY-30}}$, $bla_{\text{PER-1}}$, and $bla_{\text{GES-11}}$, respectively, by PCR. PER-1-like-derived peptides were also detected in the ADC-like-expressing isolates 20 and 22, which carry $bla_{\text{PER-1}}$ according to PCR findings (Table 1). Isolates in which no ADC-like protein or other β-lactamase with known cephalosporinase activity was detected by LC-MS/MS were susceptible to ceftazidime (Table 1), demonstrating that the detection of ADC-like protein correlated better with ceftazidime resistance than did the presence of the $bla_{\text{ADC-like}}$ gene.

In conclusion, multiple expressed β-lactamases could be identified simultaneously using LC-MS/MS. The β-lactamases most likely responsible for the resistant phenotype were detected in all isolates that were resistant to carbapenems and/or ceftazidime. The excellent correlation between the detection of the expressed β-lactamases and the resistance phenotypes demonstrated an added value for LC-MS/MS in rapid antimicrobial susceptibility testing.

Nucleotide sequence accession number. The nucleotide sequence of IS*Aba16*-$bla_{\text{OXA-64}}$ has been submitted to GenBank under accession number KP890935.

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We have no conflicts of interest to declare.

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