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Phenotypical-, biochemical- and molecular-based techniques for detection of metallo-β-lactamase NDM in *Acinetobacter baumannii*

Rémy A. Bonnin, Thierry Naas, Laurent Poirel, and Patrice Nordmann*

Service de Bactériologie-Virologie, INSERM U914 « Emerging Resistance to Antibiotics », Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine et Université Paris-Sud, K.-Bicêtre, France

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Running title: NDM-producer detection in *A. baumannii*

* Corresponding author. Mailing address: Service de Bactériologie-Virologie-Hygiène, Hôpital de Bicêtre, 78 rue de Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France. Phone: 33-1-45-21-36-32. Fax: 33-1-45-21-63-40
E-mail: nordmann.patrice@bct.aphp.fr

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Seven NDM-positive *Acinetobacter baumannii* isolates of worldwide origin were studied to evaluate the best technique for their identification. Detection of carbapenemase producers based on the measurement of carbapenemase activity by UV spectrophotometry (as for *A. baumannii* producing other types of carbapenemase), or by the modified Hodge test, failed. Inhibition activity using EDTA was a sensitive technique, but lacked of specificity as compared to molecular-based techniques which remain the gold standard.

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The emergence of the metallo-ß-lactamase (MBL) named NDM-1 (New Dehli metallo-ß-lactamase) is one of the latest and most important resistance mechanisms reported in Gram-negative rods (17). The bla_{NDM-1} gene identified initially in *Klebsiella pneumoniae* and *Escherichia coli* mostly from India and Pakistan has been recently identified extensively from other enterobacterial species worldwide (18). Besides spread of NDM gene in *Enterobacteriaceae*, reports of NDM producers in A. baumannii are increasingly identified worldwide (10-12).

Whereas detection of NDM producers in *Enterobacteriaceae* is easy (16), our preliminary results indicated that detection of NDM-producing *A. baumannii* may be much more complicated. Therefore, we evaluated several techniques for the detection of NDM-producing *A. baumannii* clinical isolates.

A collection of seven NDM-positive *Acinetobacter baumannii* isolates of worldwide origin was studied, to identify the best approach to detect the production of NDM-type carbapenemases among clinical isolates. Another collection corresponding to carbapenemase-producing but NDM-negative *A. baumannii* isolates of worldwide origin was studied for comparison (Table 1). Those latter strains produced either Ambler class B carbapenemases (IMP-4, VIM-4, or SIM-1), class D carbapenemases (OXA-23, OXA-40, or OXA-58), or a class A carbapenemase (GES-14). Susceptibility testing was performed by Etest (AB bioMérieux, Solna, Sweden) on Mueller-Hinton agar plates at 37°C, and results were classified according to the updated CLSI guidelines (3). The CLSI breakpoints of *A. baumannii* for imipenem and meropenem were as follows: susceptible (S) ≤4 µg/ml; resistant (R) ≥16 µg/ml (3). The EUCAST breakpoints for imipenem and meropenem were as follows: S ≤2 µg/ml; R ≥8 µg/ml. Whereas those for doripenem were S ≤1 µg/ml; R ≥4 µg/ml (6). All NDM-producing *A. baumannii* clinical

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isolates were resistant to all β-lactams including all carbapenems according to CLSI and EUCAST guidelines (Table 1).

The Etest MBL strip is one of the methods advocated for detection of MBLs based on inhibition of the MBL activity by EDTA (16). This Etest method showed a good sensitivity for the detection of MBL production. Susceptibility to imipenem was restored in presence of EDTA confirming the significant impact of MBL as a source of carbapenem resistance among MBL producers. However, several strains producing OXA-23 or OXA-40 but being MBL negative gave false positive results (Table 1). This showed that the intrinsic effect of EDTA on *A. baumannii* might interfer in the specificity of this test.

Another technique was therefore evaluated to detect MBL production using the same principle based on inhibition by EDTA. This technique consisted in two imipenem disks with or without 10 µl of EDTA 0.5 M (9). An increase of 10 mm of the inhibition zone diameter in the presence of EDTA was considered as a positive result. An increase between 5 and 10 mm was considered as doubtful and therefore requires further investigations. This technique gave similar results as those obtained with Etest MBL strips, with MBL producers being easily detected, except for an IMP-4-producing isolate possessing low MICs of carbapenems (MIC of imipenem at 4 mg/l) (Fig. 1). However, OXA-23 and OXA-40 producers gave some false-positive results as observed with Etest MBL strips (Table 1, Fig. 1).

The Modified Hodge test (MHT) has been widely used screening of carbapenemase activity because of the direct analysis of the carbapenemase activity. We therefore evaluated MHT as previously described (14) at a turbidity of 0.5 McFarland, using *E. coli* ATCC 25922 as indicator organism, *K. pneumoniae* BIC (producing the

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carbapenemase OXA-48) as positive control, and *K. pneumoniae* COO (a non-carbapenemase producing but carbapenem-resistant isolate) as negative control. It gave negative results for all tested NDM-producing *A. baumannii* (Table 1). VIM-, IMP- and some OXA-type producers gave weak synergistic images. This test was thus poorly sensitive and specific for detecting the carbapenemase activity of any carbapenemase producers.

Biochemical detection of NDM activity in *A. baumannii* was then analyzed. Ten ml of overnight broth cultures of *A. baumannii* isolates was centrifuged at 5,000 x g during 15 min and then sonicated in ice. Specific activities for carbapenems were measured using UV spectrophotometry at a wavelength value of 297 nm for imipenem as described (4). Detection of carbapenemase activity by UV spectrophometry performed with crude culture extracts of NDM producers did not give any significant positive result. The standard mean of specific activities obtained for the NDM-producers was evaluated at 5.7 mU/mg of proteins. This value was not significant as compared to the value obtained with the reference strain *A. baumannii* AYE strain (8) that does not possess any acquired carbapenemase gene and is susceptible to imipenem (2.6 mU/mg of proteins which correspond to the baseline). Specific activities measured using other carbapenem molecules gave similar results but lower level of carbapenemase activities (Table 1). Similar low-level activities were observed for carbapenem-hydrolyzing class D β-lactamases (CHDL) whereas high level of activity was observed for class B ß-lactamases of other types VIM, IMP, and SIM (Table 1). The latter results may be related to either stronger promoter or/and plasmid location of those MBL genes.

Molecular-based techniques performed as previously described (16), using primers NDM-For (5'-GGTGCATGCCCGGTGAAATC-3') and NDM-Rev (5'-

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ATGCTGGCCTTGGGGAACG-3') for internal gene amplification (660-bp in-size amplicon), and primers pre-NDM-for (5'-CACCTCATGTTTGAATTCGCC-3') and pre-NDM-rev (5'-CTCTGTCACATCGAAATCGC-3') for amplification of the entire gene sequence (984-bp in-size amplicon), are useful tools for identification of NDM producers and the precise identification of the resistance determinant, respectively. In a single case, as reported previously, two carbapenemase genes were identified that were $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ (20). Using these PCR-based techniques, all NDM producers were detected. Multiplex PCR for detecting several carbapenemase genes should be adapted to *A. baumannii* since recently developed multiplex PCR schemes focused on detection of carbapenemase genes only in *Enterobacteriaceae*. One of the limit of molecular-based techniques however corresponds to a lack of detection of carbapenemase producers due to unknown carbapenemase genes.

This work indicates that identification of NDM producers may be suspected by comparing results of MIC of carbapenems with or without EDTA. Detection of carbapenemase activity using either the modified Hodge test or UV spectrophotometry is likely to fail to detect NDM producers in *A. baumannii* whereas this detection is efficient for in *Enterobacteriaceae*. The discrepancy between *A. baumannii* and *Enterobacteriaceae* could be explained by a weak level enzyme production in *A. baumannii* likely related to the chromosomal location (a single copy) of the bla_{NDM} gene. Considering that many microbiology laboratories do not possess the facilities for molecular detection, carbapenem-resistant *A. baumannii* should be screened first using EDTA-inhibition based techniques following by further PCR-based techniques in reference laboratory.

These observations may have clinical implications for detection and therefore for

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126 controlling the spread of NDM producers in *A. baumannii* which is at our opinion one of
127 the most difficult task considering the persistance of multidrug resistant *A. baumannii* in
128 many health care facilities.
129
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208 Figure legend.

209 Comparison of the IPM-EDTA synergy test for carbapenemase-producing *A. baumannii*
210 clinical isolates. (A) Imipenem disk (10µg) alone. Lane 1, NDM-1-producing *A.*
211 *baumannii* (isolate SLO); lane 2, NDM-2-producing *A. baumannii* (isolate ML); lane 3,
212 IMP-4-producing *A. baumannii* (isolate IMP); lane 4, VIM-4-producing A.
213 genomospecies 16 (isolate A154); lane 5, OXA-23-producing *A. baumannii* (isolate
214 Acb2); lane 6, OXA-58-producing *A. baumannii* (isolate MAY). (B) imipenem disk
215 supplemented with 10 µl of EDTA at 0.5 M. A significant increase of the inhibition zone
216 in the presence of EDTA was considered as positive.

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Table 1. Results of MIC values with the Etest MBL, combined disk IPM/IPM+EDTA test, modified Hodge test, specific activities and PCR-technique for detection of carbapenemase producers in *A. baumannii*.

Isolates	Acquired Carbapenemase gene(s)	MIC (µg/ml)						Etest MBL	IPM/IPM+EDTA test (Diameter -/+ EDTA) (mm)	Hodge test	Specific activity (mU/mg)[a]			PCR results[c]	References
		CTX	CAZ	IPM	IPM+ EDTA	MEM	DOR				IPM	MEM	ERT		
A. baumannii SLO	NDM-1	>256	>256	>32	1	>32	>32	+	+ (6/25)	-	5.1	0.6	1.4	+	This study
A. baumannii All	NDM-1	>256	>256	>32	<1	>32	>32	+	+ (6/24)	-	5.9	0.9	1.4	+	10
A. baumannii Ora-1	NDM-1	>256	>256	>32	<1	>32	>32	+	+ (6/22)	-	6.4	0.9	1.1	+	This study
A. baumannii StN	NDM-1	>256	>256	>32	<1	>32	>32	+	+ (6/23)	-	6.3	0.8	1.2	+	This study
A. baumannii Gen	NDM-1, OXA-23	>256	>256	>32	3	>32	>32	+	+ (6/18)	-	7.2	1.1	1.5	+	20
A. baumannii ML	NDM-2	>256	>256	>32	<1	>32	>32	+	+ (6/23)	-	4.7	0.9	1.3	+	11
A. baumannii 124	NDM-2	>256	>256	>32	<1	>32	>32	+	+ (6/23)	-	4.5	0.7	1.3	+	This study
A. baumannii IMP	IMP-4	>256	>256	4	<1	8	4	+	-/+ (14/20)	-	718.1	95.6	65.2	-	5
A. genomospecies 16 A154	VIM-4	>256	>256	32	<1	32	32	+	+ (8/25)	+	279.8	45.2	33.3	-	7
A. baumannii SIM	SIM-1	>256	>256	3	<1	4	3	-/+	-/+ (21/29)	+[b]	58.3	12.6	23.2	-	15
A. baumannii 35	OXA-23	>256	>256	32	4	32	24	-/+	-/+ (9/17)	-	2.8	1.1	0.8	-	This study
A. baumannii Acb2	OXA-23	>256	>256	32	4	32	24	-/+	-/+ (11/20)	-	2.1	1.1	0.8	-	2
A. baumannii Acb13	OXA-23	>256	>256	24	4	24	16	-/+	-/+ (11/18)	-	1.9	1.2	0.9	-	2
A. baumannii LAH	OXA-40	>256	128	32	12	>32	32	-/+	-/+ (7/18)	+[b]	2.1	0.5	0.5	-	This study
A. baumannii MUZ	OXA-40	>256	256	32	12	>32	32	-/+	-/+ (7/16)	+[b]	1.6	0.6	0.4	-	This study
A. baumannii Acb19	OXA-58	>256	>256	4	2	4	4	-	- (14/18)	-	3.1	1.2	1.5	-	2
A. baumannii MAY	OXA-58	8	2	3	2	2	1	-	- (15/17)	-	2.9	1.1	0.6	-	This study
A. baumannii MAD	OXA-58	256	256	24	16	24	24	-	- (11/15)	+[b]	3.0	1.2	0.6	-	19
A. baumannii AP	GES-14	>256	>256	24	8	16	8	-	- (11/17)	+[b]	7.5	1.4	1.1	-	1

[a] Standard deviations were within 10% of the means.
[b] Weakly positive.
c PCR were performed in standards conditions with NDM-For and NDM-Rev primers .

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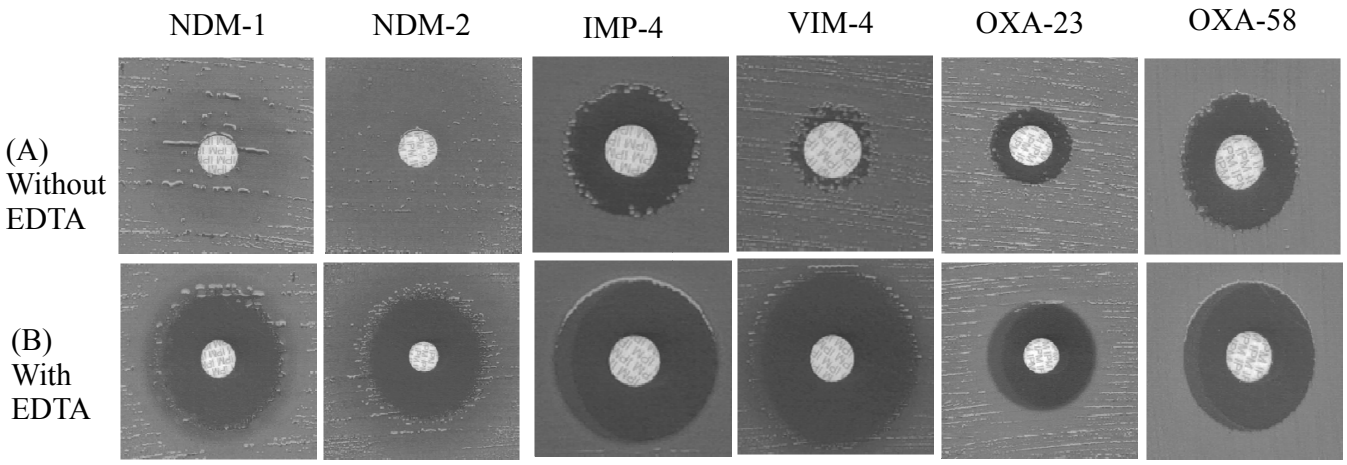
Fig. 1.



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