JCM Accepts, published online ahead of print on 23 November 2011
J. Clin. Microbiol. doi:10.1128/JCM.05247-11

Value of the Modified Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae*

Delphine Girlich, 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 Paris Sud, K.-Bicêtre, France

Keywords: OXA-48, KPC, NDM, VIM, IMP, resistance, antibiotic, carbapenemase

Corresponding author : nordmann.patrice@bct.aphp.fr

Service de Bactériologie-Virologie, hôpital de Bicêtre 78 rue du Général Leclerc, 94275 K-Bicêtre, France Tel ; 33 1 45 21 36 32

The modified Hodge test has an excellent sensitivity for detecting enterobacterial isolates producing Ambler class A (KPC) and class D (OXA-48) carbapenemases. Its sensitivity is low for NDM-1 producers (50%), but is increased to 85.7% by adding $ZnSO_4$ (100 µg/ml) in the culture medium. However, this test has a low specificity and is time-consuming.

Carbapenemase producers are increasingly reported worldwide in *Enterobacteriaceae*. Their identification is of primarily importance since carbapenemase producers are not only resistant to most (if not all) ß-lactams but also to other main classes of antibiotics. Mostly, three types of carbapenemases are now commonly identified in *Enterobacteriaceae*. They are the Ambler class A of the KPC type, the class B of the NDM-1, IMP and VIM types, and the class D of the OXA-48 type (1, 14, 15, 20, 23). Many techniques can be used for detecting production of carbapenemases, from phenotypic to advanced molecular-based techniques (13). The cloverleaf technique, or modified Hodge test (MHT), has been extensively used as a phenotypic technique for detecting carbapenemase activity (12, 13, www.cdc.gov/ncidod/dhqp/pdf/ar/HodgeTest_Carbapenemase_Enterobacteriaceae.pdf) since it is available in clinical microbiology routine settings and recommended by the CLSI (5). It is based on the inactivation of a carbapenem by carbapenemase-producing strains, that enables a carbapenem-susceptible indicator strain to extend growth towards a carbapenem-containing disk, along the streak of inoculum of the tested strain.

Since the value of MHT for detecting the currently widespread carbapenemase producers (KPC, NDM-1, OXA-48) has been poorly documented, we have initiated a study using a collection of carbapenemase- and non-carbapenemase producers with well-characterized mechanisms of resistance. Enterobacterial isolates included in our study were either resistant or of reduced susceptibility to ertapenem, according to the updated breakpoints of the CLSI guidelines (i-.e. with MIC of ertapenem ≥ 0.5 µg/ml) (5) (Table 1). The isolates produced either Ambler class A (KPC-2), class B (NDM-1 VIM-1, IMP-1), or class D (OXA-48) carbapenemases. Non-carbapenemase producers were AmpC overproducers with permeability defect, or clavulanic-acid inhibited extended-spectrum β-lactamase (ESBL) producers (mostly of the CTX-M type) with permeability defect (Table 1). Ertapenem (10 µg

disk, BioRad, Marnes-la-Coquette, France) and indicator strain *Escherichia coli* JM109 (Promega, Charbonnières-Les-Bains, France) and *E. coli* ATCC25922 were used.

Among the 35 carbapenemase producers, 24 gave positive results, 7 gave negative results and 4 gave non-interpretable results (Table 1). Class A and class D carbapenemase producers were detected by the MHT. False-negative results were obtained for 7 out of 14 NDM-1 producing *Enterobacteriaceae* (Table 1; Figure 1, panel A), which is in accordance with what had been previously observed for NDM-1 producers (4). The overall sensitivity and specificity of the MHT was low (respectively 77.4 % and 38.9%). Those non-interpretable results could correspond to isolates producing a substance, such as colicin, that may inhibit the growth of *E. coli* JM109 (Table 1). False detection of carbapenemase production was observed for 11 out of 20 isolates (Table 1). This result was in accordance with previous studies (3, 10, 13, 22).

Taking into account the high rate of false negatives among NDM producers, we tried to modify this MHT technique for improving its detection limits. Although Lee et al. suggested that a bile compound contained in MacConkey agar may improve the sensitivity of the MHT for detecting MBL producers (11), we did not observe changes in the sensitivity detection of the NDM-producers by using this medium (data not shown). As MBLs are zinc-dependent (23), zinc sulfate was added in Mueller Hinton agar (MHA) (BBL, Le Pont-de-Claix, France) at different concentrations (from 25 to 100 µg/ml). Previous studies showed that commercially-available MHA media contained concentrations of zinc varying from one- to 15-fold depending on the manufacturer (7). Cooper et al. determined zinc concentration as being 2.61 µg/ml in MHA from BBL in 1993 (7). Addition of 100 µg/ml of zinc sulfate inhibited partially growth of *E. coli* ATCC25922, giving rise to difficult interpretation of the MHT. *E. coli* JM109 was then used instead of *E. coli* ATCC25922 for growth on $ZnSO_4$-containing agar was homogeneous. Addition of zinc sulfate improved test sensitivity for 5 out

of the 7 false-negative results obtained with NDM-producers using zinc-non supplemented MHA (Table 1, Figure 1). Noticeably, two false-negative NDM-producing *E. coli* isolates remained negative despite addition of zinc sulfate (Table 1, Fig.1, panel B). As suggested for detection of the IMP- or VIM-producing *Pseudomonas aeruginosa* and *Acinetobacter* sp. (12), zinc addition improved the sensitivity of the MHT (from 77.4 to 94%) in particular with NDM-1-producing *Enterobacteriaceae* (Table 1). The effect of zinc might be multiple by increasing stability of the enzyme or/and by modifying porin expression (6). Addition of zinc sulfate did not modify the specificity of the test (38.9% with or without zinc sulfate).

This study showed that the MHT technique is highly sensitive for detecting class A, B and D carbapenemases after addition of zinc in the culture medium. However, limitations in term of clinical performance of the MHT remains its lack of specificity and the delay for obtention of the results (24 h-48 h) after isolation of a bacterial colony.

This work was funded by a grant from the INSERM (U914) and from the Ministère de l'Education Nationale et de la Recherche (UPRES-EA 3539), Université Paris XI, Paris, France, and mostly by grants from the European Community (TEMPOtest-QC, HEALTH-2009-241742). Authors report no conflict of interest.

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162

163 FIGURE LEGENDS

164 Figure. MHT on MHA (panel A), and on MHA added with zinc sulfate (100 µg/ml) (panel
165 B): 1, *E. coli* JM109; 2, *K. pneumoniae* COO (CTX-M-15 + porin loss); 3, *K. pneumoniae*
166 BIC (OXA-48); 4, *K. pneumoniae* POZ (KPC-2); 5, *E. coli* GEN (NDM-1); 6, *E. coli* RIC
167 (NDM-1); 7, *E. coli* ALL (NDM-1). Zinc sulfate improved the MHT for *E. coli* RIC and not
168 for *E. coli* ALL.

169 Table 1. Influence of $ZnSO_4$ in Mueller-Hinton agar (MHA) on the modified Hodge Test for 54 carbapenemase- and/or ESBL/AmpC-producing

170 enterobacterial isolates.

Strains	β-lactamases	MIC (µg/ml) of drug[a] :			Modified Hodge test for ETP		Reference
		IPM	ETP	MEM	MHA	MHA + ZnSO4	
Class A carbapenemases							
E. cloacae CFVL	KPC-2 + TEM-3	4	2	1	+	+	2
E. coli DIN	KPC-2 + TEM-1 + OXA-1	1	>32	2	+	+	2
E. coli PSP	KPC-2 + TEM-1 + OXA-1	0.5	0.5	0.5	+	+	2
E. coli COL	KPC-2 + TEM-1 + CTX-M-9	4	4	2	+	+	This study
K. pneumoniae COL	KPC-2 + TEM-1 + SHV-1 + CTXM-15	4	4	2	+	+	This study
K. pneumoniae KAM	KPC-3 + TEM-1	8	12	2	+	+	This study
Class B carbapenemases							
K. pneumoniae UK	NDM-1 + CTX-M-15 + CMY-4 + OXA-1	> 32	> 32	> 32	+	+	15
K. pneumoniae 6759 GEN	NDM-1 + TEM-1 + SHV-11 + CTX-M-15 + CMY-16 + OXA-1 + OXA-9 + OXA-10	12	> 32	> 32	+	+	21
K. pneumoniae 2OMA	NDM-1 + OXA-1 + SHV-11	1.5	6	2	+	+	15
K. pneumoniae IND	NDM-1 + TEM-1 + SHV-28 + CTX-M-15 + CMY-6 + OXA-1 + OXA-9	1	8	4	-	+	This study
K. pneumoniae 1OMA	NDM-1 + TEM-1 + SHV-11 + SHV-28 + CTX-M-15 + OXA-1 + OXA-9	> 32	> 32	> 32	+	+	15

K. pneumoniae 7AFR	NDM-1 + TEM-1 + SHV-28 + CTX-M-15 + CMY-6 + OXA-1	> 32	> 32	> 32	-	+	15
E. coli 5649 GEN	NDM-1 + OXA-1 + CMY-30 + TEM-1	8	> 32	12	-	+	21
E. coli RIC	NDM-1 + TEM-1 + OXA-1 + OXA-10 + CMY-16	1	3	1	-	+	This study
E. coli GUE	NDM-1 + TEM-1 + OXA-1	3	3	2	-	-	15
E. coli 271 AUS	NDM-1 + TEM-1 + CTX-M-15	6	32	16	+	+	19
E. coli ALL	NDM-1 + TEM-1 + OXA-1 + OXA-2 + CTX-M-15	4	> 32	8	-	-	16
E. coli IR5 TW	NDM-1 + TEM-1 + CTX-M-15	16	>32	16	ND[d]	+/ND	15
E. cloacae IR38	NDM-1 + CTX-M-15	2	16	2	+	+	15
P. stuartii PS1	NDM-1 + TEM-1 + OXA-1 + CMY-6	12	0.38	1.5	-	+	18
E. coli MAD	VIM-1 + CTX-M-3	1.5	0.38	0.5	+	+	2
K. pneumoniae MAD	VIM-1 + CTX-M-3	1	0.5	1	ND	ND	2
K. pneumoniae DIH	VIM-19 + TEM-1 + SHV-1 + CTX-M-3	8	16	4	ND	+/ND	This study
E. coli JAP	IMP-1	0.5	3	0.5	+	+	2
K. pneumoniae TUR	IMP-1 + SHV-5	1	2	8	ND	ND	2
Class D carbapenemases							
K. pneumoniae BIC	OXA-48	0.5	2	0.5	+	+	2
K. pneumoniae BEL	OXA-48	1	4	1	+	+	2
K. pneumoniae LIB	OXA-48	> 16	> 16	> 16	+	+	2
K. pneumoniae CHA	OXA-48 + TEM-1	0.38	1	0.5	+	+	This study
K. pneumoniae EGY	OXA-48 + CTX-M-15	2	3	2	+	+	2
E. cloacae TUR	OXA-48 + SHV-5	0.5	0.5	0.5	+	+	2
E. coli HAN	OXA-48 + CTX-M-15	3	16	1	+	+	This study
E. coli BOU	OXA-48 + CTX-M-15	0.5	0.75	0.125	+	+	This study
E. coli BER	OXA-48 + CTX-M-15	0.38	1.5	0.19	+	+	This study
E. coli AME	OXA-48 + TEM-1 + CTX-M-24	0.25	0.5	0.19	+	+	17

AmpC +/- reduced permeability							
P. mirabilis PMA	ACC-1	0.25	0.12	0.12	Weak Pos	Weak Pos	9
E. coli ECA	ACC-1	0.12	0.12	0.12	Weak Pos	Weak Pos	9
K. pneumoniae KDH	DHA-2	0.12	0.5	0.12	Weak Pos	Weak Pos	8
E. coli MET	ESAC	0.12	0.12	0.12	ND	ND	This study
E. coli Ec13 SYD	CMY-2	0.12	0.12	0.12	ND	ND	This study
E. coli MAR[b]	AmpC	16	>32	2	-	-	This study
E. cloacae BLA[b]	AmpC	0.12	1	0.12	-	-	This study
E. cloacae CON[b]	AmpC	0.12	1	0.12	+	+	This study
E. cloacae AZA[b]	AmpC	0.5	1	0.12	+	+	This study
E. cloacae POG[b]	AmpC	4	1.5	2	+	+	This study
ESBL +/- reduced permeability							
K. pneumoniae COO[c]	CTX-M-15 + SHV28	4	>32	4	-	-	This study
K. pneumoniae BER[c]	TEM-1 + SHV-28	1	4	1	-	-	This study
K. pneumoniae 648236[c]	SHV-2a	0.25	2	0.38	Weak Pos	-	This study
K. pneumoniae MEK[c]	CTX-M-15 + SHV-11	1.5	>32	6	Weak Pos	-	This study
K. pneumoniae SIM[c]	CTX-M-15 + TEM-1 + SHV-1	3	>32	3	-	-	This study
K. pneumoniae BED[c]	CTX-M-15 +TEM-1 + SHV-1	1.5	>32	4	Weak Pos	Weak Pos	This study
K. pneumoniae SHM[c]	CTX-M-15 +TEM-1 + SHV-11	3	>32	1	-	Weak Pos	This study
K. pneumoniae FOS[c]	CTX-M-15 + TEM-1 + SHV-11	6	>32	>32	+	Weak Pos	This study
K. pneumoniae LEG[c]	CTX-M-15 + TEM-1 + SHV-12	0.75	>32	3	-	-	This study

[a] Abbreviations : IMP, imipenem; ETP, ertapenem; MP, meropenem.
[b] Reduced susceptibility to ertapenem due to overexpressed AmpC
[c] Reduced susceptibility to ertapenem due to porin deficiency.
[d] ND, not determinable, due to inhibition of growth of the *E. coli* JM109 strain along the tested isolate.

Fig. 1.