Triton Hodge Test: improved protocol for Modified Hodge Test for enhanced detection of NDM and other carbapenemase producers

Fernando Pasteran[1], Lisandro J. Gonzalez[2], Ezequiel Albornoz[1], Guillermo Bahr[2], Alejandro J. Vila[2] and Alejandra Corso[1]

[1]*Servicio Antimicrobianos, Laboratorio Nacional y Regional de Referencia en Antimicrobianos, Instituto Nacional de Enfermedades Infecciosas (INEI), ANLIS "Dr. Carlos G. Malbrán". Ciudad Autónoma de Buenos Aires, Argentina.*

[2]*Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Santa Fé, Argentina*

* Corresponding author: Alejandra Corso

Address: Servicio Antimicrobianos, Departamento Bacteriologia, Instituto Nacional de Enfermedades Infecciosas-ANLIS "Dr. Carlos G. Malbrán", Av. Velez Sarsfield 563 (1282AFF), Ciudad Autónoma de Buenos Aires, Argentina

Phone/Fax: 5411 4303 2812

E-mail: acorso@anlis.gov.ar

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ABSTRACT

Accurate detection of carbapenemase-producing Gram-negative bacilli is of utmost importance to control nosocomial spread and initiate appropriate antimicrobial therapy. The Modified Hodge Test (MHT), a carbapenem inactivation assay, has shown a poor sensitivity in detecting the worldwide spread New Delhi metallo-ß-lactamase (NDM). Recent studies demonstrated that NDM is a lipoprotein anchored to the outer membrane in Gram-negative bacteria, unlike all other known carbapenemases. Here we report that membrane anchoring of ß-lactamases precludes detection of carbapenemase activity by the MHT. We also show that this limitation can be overcome by addition of Triton X-100 during the test, which elicits detection of NDM. We propose an improved version of the assay, called Triton Hodge Test (THT), which allows detection of membrane-bound carbapenemases by addition of this nonionic surfactant. This test was challenged with a panel of 185 clinical isolates (145 carrying known carbapenemase encoding genes and 40 non-carbapenemase producers). The THT displayed a test sensitivity of >90% against NDM-producing clinical isolates, improving at the same time the performance against other carbapenemases. Ertapenem provides the highest sensitivity (97 to 100% depending on the type of carbapenemase) followed by meropenem (92.5 to 100%). Test specificity was not affected by the addition of Triton (87.5% and 92.5% for ertapenem and meropenem, respectively). This simple, inexpensive test confers a large improvement to the sensitivity of MHT for the detection of NDM and other carbapenemases.

Detection of carbapenemase producers in the clinical laboratory is of major importance to define an appropriate empiric antimicrobial therapy and implement infection control measures. Acquired carbapenemases belong to three of the four known classes of ß-lactamases, namely, Ambler Class A (KPC, Sme, NMC-A, IMI-1, and some allelic variants of GES), Ambler Class B or metallo-ß-lactamases (MBLs) (VIM, IMP, NDM, SPM, etc) and Ambler Class D or oxacillinases (OXAs) (OXA-48, OXA-181, etc) (1).

The modified Hodge test (MHT) is a phenotypic screening test to identify carbapenemase producers, being recommended by Clinical and Laboratory Standards Institute (CLSI) for *Enterobacteriaceae* with elevated carbapenem MICs or reduced disk diffusion inhibition zones (2). This test is based on the inactivation of a carbapenem by carbapenemase-producing strains that enable a susceptible indicator strain to extend growth toward a disk containing this antibiotic, along the streak of inoculum of the tested strain. The MHT has shown an excellent sensitivity in the detection of Class A and Class D carbapenemase producers (3-6). Unfortunately, the MHT performs poorly in the detection of NDM-producing isolates, with a sensitivity below 50% (3-7). As NDMs are Zn(II) dependent enzymes, it has been suggested that the deficit of this cation in commercial media could be responsible for these false negative results (4). Indeed, Zn(II) availability has been shown to be crucial for bacterial fitness when resistance to antibiotics depends on Class B enzymes (8). However, supplementation of culture media with up to 100 µg/ml of zinc sulfate failed to reverse these false negative results (4, 9), suggesting the presence of other mechanisms responsible for this deficient performance.

Recent experiments have shown that NDM-1 is a lipoprotein anchored to the outer membrane in Gram-negative bacteria, unlike all other known carbapenemases, characterized as soluble periplasmic enzymes (10, 11). This cellular localization is

consistent with the presence of a canonical lipidation sequence (LSGC), called lipobox, proximal to the signal peptide of NDM-1 (and all NDM variants) (10). In this work, we show that false negative results with the MHT can be attributed to membrane anchoring of NDM. We propose a simple improvement of the MHT, called Triton Hodge Test (THT), which elicits detection of these membrane-bound carbapenemases by addition of a nonionic surfactant during the test.

Material and Methods

Bacterial isolates

i. Isogenic *Escherichia coli* DH5α strains.

Isogenic *E. coli* DH5α strains harboring native and chimeric variants of the NDM-1 and VIM-2 genes with different cellular localizations were used to explore the impact of membrane anchoring of MBLs in the performance of the MHT. *E. coli* DH5α was used for expression of plasmid pMBLe, containing $bla_{\text{NDM-1}}$ or $bla_{\text{VIM-2}}$, which retain the native peptide leader of each ß-lactamase (see below). MBL mutants NDM-1 C26A (NDM-1 in which lipobox was disrupted by replacing Cys by Ala at the indicated position), V-NDM-1 (NDM-1 in which the first 47 amino acids were replaced by the first 42 residues of VIM-2) and N-VIM-2 (VIM-2 in which the first 42 amino acids were replaced by the first 47 residues of NDM-1) were constructed and sub-cloned into plasmid pMBLe (see below). These plasmids allow expression of membrane-bound variants: native NDM-1 and N-VIM-2, and soluble periplasmic variants: native VIM-2, NDM-1 C26A and V-NDM-1 (11).

Briefly, full-length $bla_{\text{NDM-1}}$ and $bla_{\text{VIM-2}}$ (including their native peptide leaders) were amplified using the following primers and subcloned into *Nde*I and *Hind*III sites of pMBLe: NDM1*Nde*I$_{\text{Fw}}$ (5' TATACATATGGAATTGCCCAATATTATGCACC 3'),

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NDM1*Hind*III$_{Rv}$ (5' GACGTAAGCTTCTAGCGCAGCTTGTCGGC 3') for *bla*$_{NDM-1}$; VIM2*Nde*I$_{Fw}$ (5' GACATCATATGTTCAAACTTTTGAGTAAGTTATTGGTC 3'), VIM2*Hind*III$_{Rv}$ (5' GACGTAAGCTTCTACTCAACGACTGAGCGATTTGTG 3') for *bla*$_{VIM-2}$. All PCRs were carried out using Platinum® Pfx DNA Polymerase (Invitrogen) with the following thermal cycle: 3 min at 95°C, 30 cycles of 15 s at 95°C, 30 s at 55°C and 1 min at 68ºC, and 10 min at 68ºC. NDM-1 C26A mutant gene was generated from pMBLe-*bla*$_{NDM-1}$ by site-directed mutagenesis, as described (12), using primers NDM-1-C26A$_{Fw}$ (5' CATTGATGCTGAGCGGGGCGATGCCCGGTGAAATC 3') and NDM-1-C26A$_{Rv}$ (5' GATTTCACCGGGCATCGCCCCGCTCAGCATCAATG 3'). V-NDM-1 and N-VIM-2 were constructed by overlap extension PCR using overlapping primers VIM2-B (5' ATTCGGTGCGAGCTGGCGGAAAACCAGATCCCCGACCGGAATTTCGC 3'), NDM1-C (5' GATCTGGTTTTCCGCCCAGCTCGCACCG 3'), NDM1-D (5' ACCATCGGCAATCTGGTAAAGCCGGACCTCGCCAAACCGTTGGTCGCC 3'), VIM2-E (5' GAGGTCCGGCTTTACCAGATTGCCG 3') with external primers VIM2*Nde*I$_{Fw}$, NDM1*Nde*I$_{Fw}$, VIM2St*Hind*III$_{Rv}$ and NDM1St*Hind*III$_{Rv}$. All constructs were verified by DNA sequencing (University of Maine, USA). Plasmid pMBLe was subsequent introduced into *E. coli* DH5α as previously described (13).

ii. Panel of clinical isolates. A total of 185 clinical isolates were included (145 carrying known carbapenemase encoding genes and 40 isolates without carbapenemase production). The carbapenemases represented were: Class A carbapenemases (KPC-2, KPC-3, GES-3, GES-5, NMC-A and Sme-1b, $n = 25$), Class B carbapenemases (NDM-1, IMP-1, IMP-8, IMP-13, IMP-16, IMP-18, SPM-1, VIM-1, VIM-2, and VIM-11, $n = 100$) and Class D carbapenemases (OXA-48, OXA-163, OXA-181, OXA-247 and OXA-438, $n = 20$). Strains were isolated from clinical specimens, as follow: urine

(35%), blood (35%), respiratory tract (15%) and other sites such as bone, abdominal, CSF, etc (15%). Only a single isolate per patient was included in the panel. The isolates, belonging to the collection of the National and Regional Reference Laboratory (INEI) for the Latin American region, correspond to submissions of very diverse locations (21 countries, 1226 labs surveyed from 2010 to 2014) and thus are expected to display minimum clonal and enzyme bias. When PFGE studies were available (14), strains included were non-clonal. Panel isolates were previously identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex MALDI-TOF; Bruker, Germany). Only those strains which met the score cutoffs recommended by the manufacturer used to determine species-level identification (≥2.000) and showed a ≥10% score difference between the first two best matches in the database (15), were included in this study. *Proteus* spp. isolates were excluded due to their frequent swarming during the assays, which prevented interpretation of the phenotypic confirmatory tests.

Antimicrobial susceptibility testing

The minimum inhibitory concentration of imipenem, meropenem and ertapenem was determined by the broth microdilution method (in-house-prepared panel), according to CLSI guidelines (2).

Characterization of the mechanisms of resistance.

PCR analysis followed by DNA sequencing of the amplicons were considered the gold standards for characterization of the β-lactamases. Strains were analyzed for bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{KPC}, $bla_{OXA\text{-}48\text{-}like}$, bla_{Sme}, $bla_{IMI/NMC\text{-}A}$, bla_{GES}, $bla_{CTX\text{-}M}$, bla_{PER} and bla_{CMY} as described previously (16, 17). Outer membrane porin profiles of carbapenem non-susceptible, carbapenemase nonproducers were determined by SDS-

PAGE (18, 19). Overexpression of chromosomal AmpC was evaluated by spectrophotometric analyses, as described (20).

Cell fractionation and NDM-1 detection

Lysogeny broth media (25-mL) was inoculated with *E. coli* DH5α pMBLe NDM-1, *Providencia rettgeri* 15758, *Serratia marcescens* 17468 or *Enterobacter cloacae* 17464, and grown with shaking at 37ºC until OD600nm 1 (in the case of *E. coli* DH5α expression of NDM-1 was induced at OD600nm 0.4 by addition of 50 µM IPTG). Cells were pelleted, resuspended in HEPES 10 mM, NaCl 200 mM, PMSF 1 mM pH 7.4, and disrupted by sonication. Cell debris was then removed by centrifugation at 14,000xg and 4ºC for 20 minutes, and total protein concentration determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific). Equal amounts of cleared homogenates (5 mg total protein) were subjected to ultracentrifugation at 150,000xg and 4ºC for 1 h. Membrane (pellet) and soluble fractions (supernatant) were separated and concentrated 5X for electrophoresis. NDM-1 protein was detected in bacterial fractions by SDS-PAGE followed by Western blot with polyclonal antibodies against NDM-1 (kindly provided by Prof. Robert Bonomo, Case Western Reserve University, Cleveland, OH) and immunoglobulin G-alkaline phosphatase conjugates.

Phenotypic Confirmatory Assays

i. MHT. The MHT was performed as described previously (2). Briefly, a 1/10 dilution of an inoculum of the indicator organism *E. coli* ATCC 25922, adjusted to a 0.5 McFarland turbidity standard, was used to inoculate the surface of plates containing Mueller-Hinton agar (MHA) (Becton Dickinson, BBL) by swabbing. After the plates were allowed to stand for 10 min at room temperature, disks (BBL) containing meropenem (10 µg) or ertapenem (10 µg) were placed onto the agar plates. Subsequently, three to five colonies of the test organisms (from an agar plate grown

overnight) were inoculated onto the plate in a straight line out from the edge of the disk, using a 10-ul loop. Plates were examined after overnight incubation at 35ºC. For carbapenem hydrolysis screening, growth of the indicator strain towards the carbapenem disk was interpreted as a positive or weak positive result, depending on the magnitude of the enhanced growth (measured with a ruler, as described [21]). Isolates allowing growth of the indicator strain up to 3 mm were recorded as "weak positive", while those with > 3 mm were labeled as "positive". The choice of this cutoff was based on the fact that most discrepancies in result interpretation occur, from our experience, in cases where growth of the indicator strain is less than 3 mm. The absence of growth of the indicator strain toward the carbapenem disks was interpreted as a negative result. For test isolates that produced substances which inhibited growth of the indicator strain (a clear area was seen around the streak), the MHT was recorded as "uninterpretable". Two laboratory staff read all test results independently (discrepancies were solved by a third observer). Figure 1 illustrates the components of the MHT.

ii. Triton Hodge Test. For solubilization of membrane proteins, the MHT was performed on a MHA plate (Becton Dickinson, BBL) flooded with 50 µl of pure Triton X-100 reagent (0.2% v/v in the MHA plate). Briefly, the detergent was dripped in the center of the plate and quickly distributed by streaking 4 to 6 times a swab over the entire sterile agar surface until complete absorption. Delays of more than 10 minutes in streaking the Triton X-100 might alter the agar surface around the Triton X-100 drop. Flooded plates were stored at 4°C until use. Before inoculation with the indicator organism, excess surface moisture was removed by evaporation at 35ºC. *E. coli* DH5α laboratory strains were included as controls in each THT assay performed. In addition to meropenem and ertapenem, we used imipenem, a substrate not included in CLSI recommendations for the MHT (2), in the standardization of the THT with the final aim

of looking for the optimal test conditions. However, imipenem showed more false positives than other carbapenems in preliminary assays, and therefore was excluded from further analysis. The THT was also challenged with non-*Enterobacteriaceae* isolates, which are not included in the CLSI recommendations for MHT (2). Test interpretation was performed as defined for the MHT (see above).

MHA used in the phenotypic confirmatory asssays

A method comparison between MHA batches with different Zn(II) concentration was performed with isolates expressing different MBLs. We included a batch of MHA from Laboratorios Britania (Argentina) with a Zn(II) concentration of 14.6 ± 0.5 p.p.m. w/w in the dehydrated media or 0.54 to 0.57 µg/ml in the hydrated media (as declared in the certificate of analysis performed by atomic absorption - acetylene flame by a reference university lab at Physics School, Faculty of Pharmacy and Biochemistry, University of Buenos Aires), and the reference BD/BBL batch, recommended as reference MHA for MBL detection (20), with a Zn(II) concentration of 23.5 ± 0.5 p.p.m. w/w in the dehydrated media or 0.87 to 0.91 µg/ml in the hydrated media, according to data provided by the reference university lab.

Triton plate stability assay

Triton-flooded agar plates were stored in sealed packages at 4ºC and examined every two weeks with one NDM-1-producing *P. rettgeri* isolate, one OXA-48-producing *E. coli* isolate, one KPC-2-producing *Klebsiella pneumoniae* isolate and one carbapenemase nonproducer (a CTX-M-15-producing *K. pneumoniae* clinical isolate).

RESULTS

Membrane anchoring of MBLs gives rise to a false negative MHT

NDM-1 is bound to the outer membrane in its native form. In order to explore the impact of MBL membrane anchoring in the performance of the MHT, we tested *E. coli* DH5α strains with soluble and membrane-anchored variants of NDM-1 and VIM-2. We challenged the MHT with an *E. coli* DH5α strain expressing membrane–bound NDM-1 and two *E. coli* DH5α strains expressing soluble (periplasmic) variants of NDM-1: NDM-1 C26A, containing a mutation on the lipidation site that precludes membrane anchoring, and V-NDM-1, a chimera of NDM-1 and the N-terminal peptide leader of VIM-2, which also gives rise to a soluble enzyme. As shown in Figure 1a, the MHT was negative for membrane–bound NDM-1 but was positive for both *E. coli* cells expressing the soluble NDM-1 variants. To further validate these findings, we tested an *E. coli* DH5α strain expressing VIM-2 in its native, soluble form, compared to an isogenic strain expressing N-VIM-2. N-VIM-2 is a membrane-anchored variant of VIM-2 resulting from replacement of the native signal peptide of VIM-2 by that of NDM-1, including the lipidation site. The strain expressing soluble VIM-2 gave a clear positive MHT. Conversely, the strain expressing the chimeric membrane-anchored N-VIM-2 presented a negative MHT (Fig. 1a). These experiments clearly show that membrane anchoring of ß-lactamases to the bacterial membrane gives rise to a false negative MHT.

Detaching membrane-bound MBLs with a nonionic surfactant improves MHT performance

We aimed to solubilize NDM-1 by addition of a nonionic detergent. We tested *E. coli* DH5α strains expressing soluble and membrane-anchored variants of NDM-1 and VIM-2 in MHA plates previously treated with Triton X-100. Carbapenemase-like patterns were clearly observed in *E. coli* DH5α strains expressing the membrane-bound NDM-1 and N-VIM-2 chimera upon addition of Triton (Fig. 1b), in contrast to the negative

results observed in the absence of detergent (Fig. 1a). As expected, isogenic *E. coli* DH5α strains expressing the soluble enzymes VIM-2, NDM-1 C26A variant and V-NDM-1 also tested positive for the MHT in the presence of detergent (Fig 1b). These results suggest that addition of a nonionic detergent into the test plate can revert the false negative MHT observed for membrane-bound ß-lactamases, probably by release of the lipid anchor. We propose naming this modification as Triton Hodge Test (THT).

Cellular localization of NDM-1 in clinical isolates

We evaluated the cellular localization of NDM-1 in different species of clinical isolates to validate the generality of this approach. Indeed, NDM-1 was detected in the membrane fraction of *Providencia rettgeri*, *Serratia marcescens* and *Enterobacter cloacae* clinical isolates (Figure 2), as observed for the model *E. coli* DH5α strain. Instead, no traces of NDM-1 could be detected in the soluble, periplasmic fraction of any of these strains. *P. rettgeri* M15758 showed visibly lower amounts of NDM-1 compared to the other two tested strains, as evidenced in both whole cells and membrane fractions. Among these strains, only *E. cloacae* M17464 tested positive (indentation <3 mm) for carbapenemase activity by the MHT (Table 1). These results encouraged us to evaluate the use of Triton to improve NDM detection in clinical strains.

Comparative performance of the MHT and THT for carbapenemase detection among clinical isolates

We challenged the MHT and the THT with a panel of clinical isolates with distinct susceptibility profiles to carbapenems. Figure 3a shows the performance of the THT and the MHT for representative NDM-producers. The MHT performs poorly in the detection of NDM-producing isolates (20% and 32.5% sensitivity for meropenem and ertapenem, respectively) (Table 1). Most NDM producers with a positive MHT showed

weak enhanced growth (indentation <3 mm) of the indicator strain (Table 1). In contrast, the sensitivity of THT was 100% with ertapenem and 92.5% with meropenem for NDM-1 producing strains (false negative results corresponded to *Providencia* spp. isolates) (Table 1). Surprisingly, the THT also performed better than the MHT for organisms producing other types of MBLs, especially among *Pseudomonas* spp. isolates (Table 2 and Fig. 3b). Only one IMP-13-producing *P. aeruginosa* isolate showed an uninterpretable result in the Triton test. The THT showed a performance comparable to the MHT against Class A and Class D enzymes (Table 2). Overall, the sensitivity of THT for carbapenemase detection was 97% (141 positives out of 145) to 99% (143/145), for meropenem and ertapenem respectively, compared to 67% (97/145) to 72% (105/145) of the MHT.

The MHT has been largely associated with false positive results among ESBL/AmpC producers (20, 23). Thus, we challenged the THT with a panel of carbapenem-resistant, non-carbapenemase producers clinical isolates. The THT displayed a false positive rate similar to the MHT (Table 2). The highest specificity was observed when using meropenem, a stable substrate for AmpC/CTX-M enzymes (false positive results were one *K. pneumoniae* CTX-M-2 producer and one *E. cloacae* AmpC hyper-producer that already had a positive MHT, and one CTX-M-2-producing *K. pneumoniae* isolate). False positives results with ertapenem included the former strains and two additional *E. cloacae* isolates producing either CTX-M or AmpC.

Triton-flooded plates stored up to 12 weeks showed similar results with the control strains. The studies were not continued beyond this time because MH storage is not recommended for longer periods, according to the manufacturer guidelines.

Effect of Zn content of growth media on test performance

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We compared the test performances using reference media and an alternative commercial MHA brand with different zinc contents. The proportion of NDM-1 producers that tested positive in this alternative media, containing 0.54 to 0.57 µg/ml of Zn(II), paralleled the results obtained with reference MHA agar with 0.87 to 0.91 µg/ml of Zn(II). The MHT displayed 20% and 32.5% of positive results for meropenem and ertapenem, respectively, while values for the THT were of 92.5% (meropenem) and 100% (ertapenem). The size of enhanced growth of the indicator strain was similar regardless of the MHA batch. Clinical isolates producing other types of MBL showed equivalent results in both MHA (not shown).

DISCUSSION

Effective screening of carbapenemase producers in clinical microbiology laboratories requires the development of sensitive and inexpensive methods. The widespread MHT fails in detecting NDM-1 producers (3-7, 9). Here we show that these false negative results are due to the fact that NDM-1 is a membrane-bound lipoprotein, and that, in contrast with previous suggestions (22, 24), the Zn(II) levels in commercial media do not sensibly affect detection of NDM producers.

NDM-1 is a membrane-anchored lipoprotein associated to the outer membrane by a lipid moiety covalently bound to a Cys residue (10, 11). This feature is common to all NDM variants, making them different from the rest of periplasmic, soluble metal-dependent carbapenemases, such as VIM-2. Here we also show that NDM-1 is bound to the membrane in clinical strains, being absent in the soluble fraction. Engineered soluble variants of NDM-1 (i.e., not membrane bound) can be detected by the MHT, as opposed to membrane-anchored variants. These results suggest that the membrane-bound nature of NDM precludes carbapenemase detection by the MHT, by preventing

the release of the enzyme into the extracellular medium. Indeed, all clinical strains producing NDM-1 with a negative MHT became positive upon addition of the nonionic surfactant Triton X-100, able to solubilize membrane lipoproteins (25-28). These results are consistent with other reports suggesting that Triton X-100 is able to release NDM-1 from membranes of different bacteria while preserving β-lactam activity (Gonzalez L. et al., submitted for publication).

We propose the addition of a nonionic surfactant as a simple and inexpensive strategy to improve the performance of the MHT for detection of NDM producers. This modification, named the Triton Hodge Test (THT), also improves detection of organisms producing other soluble class B enzymes, while not affecting detection of Class A and D producers. This is possibly due to an enhanced periplasmic release of the soluble ß-lactamases. The enhanced detection was also observed in bacteria not currently included in CLSI recommendations for MHT, such as the non-*Enterobacteriaceae*, in which the number of uninterpretable results, largely associated to this group, was significantly reduced. Thus, the THT represents an attractive alternative to other methods (29). On the other hand, the occurrence of false detection of carbapenemase production using this approach among isolates with reduced carbapenem susceptibility due to dual mechanisms (ESBLs/AmpC plus decreased porins) was similar to that observed for MHT. Therefore, for areas with high prevalence of these types of strains, the positive predictive value of THT would be low, paralleling that of the MHT (20, 23).

Ertapenem is the best substrate to screen carbapenemase producers among *Enterobacteriaceae*. Indeed, it was the only compound enabling carbapenemase detection in *Providencia* spp. isolates (which show a lower endogenous expression of NDM-1 and lower MIC values). The use of this carbapenem, however, might increase

false positives from carbapenemase nonproducers, as we report in tests with *Klebsiella* and *Enterobacter* isolates. The use of meropenem as a second substrate, if possible, is an alternative approach to reduce the number of isolates that could require further confirmation by other methods. Among non-*Enterobacteriaceae*, both carbapenems had almost identical results, except for an IMP-18 producing strain, which was detected only with meropenem.

Based on our results, we provide additional recommendations to clinical microbiology laboratories aimed to improve routine detection of NDM and other carbapenemase producers with the THT: (i) The long-term stability of Triton-flooded plates (up to 12 weeks) enables early preparation and fractionation in aliquots as an efficient alternative to daily on-site plate preparation; (ii) The THT can be performed with MHA from commercial sources proposed as reference for MBL detection (22), such as Becton Dickinson BBL media, but also from other commercial manufactures with adequate Zn(II) levels to ensure MBL activity ($\geq$ 0.54 µg/ml in hydrated media).

The present study demonstrates that addition of the nonionic surfactant Triton X-100 to the MHT represents a simple and non-expensive variant of this popular test that allows NDM-1 detection and, at the same time provides better sensitivity for isolates producing other carbapenemases. These features make it a good candidate as a diagnostic tool for routine laboratories.

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Conflict of interests:

No conflict of interests is declared.

References

1. **Bush K, Jacoby G.** 2010. Updated functional classification of β-lactamases. Antimicrob. Agents Chemother. **54:**969–976.
2. **Clinical and Laboratory Standards Institute.** 2015. Performance standards for antimicrobial susceptibility testing: twenty-fifth informational supplement M100-S25. CLSI, Wayne, PA, USA.
3. **Doyle D, Peirano G, Lascols C, Lloyd T, Church DL, Pitout JD.** 2012. Laboratory detection of *Enterobacteriaceae* that produce carbapenemases. J. Clin. Microbiol. **50:**3877-3880.
4. **Girlich D, Poirel L, Nordmann P.** 2012. Value of the modified Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae*. J. Clin. Microbiol. **50:**477-479.
5. **Castanheira M, Deshpande LM, Mathai D, Bell JM, Jones RN, Mendes RE.** 2011. Early dissemination of NDM-1- and OXA-181-producing *Enterobacteriaceae* in Indian hospitals: report from the SENTRY Antimicrobial Surveillance Program, 2006-2007. Antimicrob. Agents Chemother. 55:1274-1278.
6. **Saito R, Koyano S, Dorin M, Higurashi Y, Misawa Y, Nagano N, Kaneko T, Moriya K**. 2015. Evaluation of a simple phenotypic method for the detection of carbapenemase-producing *Enterobacteriaceae*. J. Microbiol. Methods. **108:**45-8.
7. **Bonnin RA, Naas T, Poirel L, Nordmann P**. 2012. Phenotypic, biochemical, and molecular techniques for detection of metallo-β-lactamase NDM in *Acinetobacter baumannii*. J. Clin. Microbiol. **50:**1419-1421.

8. **Meini M, Tomatis P, Weinreich D, Vila AJ.** 2015. Quantitative description of a protein fitness landscape based on molecular features. Mol. Biol. Evol. **32**: 1774-87.
9. **Kim HK, Park JS, Sung H, Kim MN.** 2015. Further modification of the modified Hodge test for detecting metallo-β-lactamase-producing carbapenem-resistant *Enterobacteriaceae*. Ann. Lab. Med. **35:**298-305. doi: 10.3343/alm.2015.35.3.298.
10. **King D, Strynadka N.** 2011. Crystal structure of New Delhi metallo-ß-lactamase reveals molecular basis for antibiotic resistance. Protein Sci. **20:**1484-91.
11. **Gonzalez L, Bahr G, Bonomo RA, Vila A.** 2014. Membrane anchoring of carbapenemase NDM-1 favors protein stability and resistance transfer. In: Abstracts of the Fifty-four Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington DC, USA, 2014. Abstract C-162c. American Society for Microbiology.
12. **Moran-Barrio J, Lisa M, Vila A.** 2012. In vivo impact of Met221 substitution in GOB metallo-β-lactamase. Antimicrob. Agents Chemother. **56:**1769-1773.
13. **Choi K, Kumar A, Schweizer H**. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64:**391-397.
14. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233-9.

15. **Almuzara M, Barberis C, Traglia G, Famiglietti A, Ramirez MS, Vay C**. 2015 Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry for species identification of nonfermenting Gram-negative bacilli. J. Microbiol. Methods **112:**24-7.

16. **Pasteran F, Veliz O, Ceriana P, Lucero C, Rapoport M, Albornoz E, Gomez S, Corso A, ReLAVRA Network Group**. 2015. Evaluation of the blue-carba test for rapid detection of carbapenemases in gram-negative bacilli. J. Clin. Microbiol. **53:**1996-8.

17. **Melano R, Corso A, Petroni A, Centron D, Orman B, Pereyra A, Moreno N, Galas M.** 2003. Multiple antibiotic-resistance mechanisms including a novel combination of extended-spectrum ß-lactamases in a *Klebsiella pneumoniae* clinical strain isolated in Argentina. J. Antimicrob. Chemother. **52:**36–42.

18. **Martínez-Martínez L, Hernández-Allés S, Abertí S, Tomás J, Benedi V, Jacoby G.** 1996. In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded- espectrum cephalosporins. Antimicrob. Agents Chemother. **40:**342–348.

19. **Hamzehpour M, Pecher J, Plesiat P, Kohler T.** 1995. OprK and OprM define two genetically distinct multidrug efflux systems in *Pseudomonas aerugi*nosa. Antimicrob Agents Chemother **39:**2392-2396.

20. **Pasteran F, Mendez T, Rapoport M, Guerriero L, Corso A**. 2010. Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class a carbapenemase in species of *Enterobacteriaceae* by incorporating boronic acid. J. Clin. Microbiol. **48:**1323-1332.

21. **Ribeiro VB, Linhares AR, Zavascki AP, Barth AL**. 2014. Performance of quantification of Modified Hodge Test: an evaluation with *Klebsiella*

pneumoniae carbapenemase-producing *Enterobacteriaceae* isolates. Biomed. Res. Int. doi: 10.1155/2014/139305.

22. **Dortet L, Bréchard L, Poirel L, Nordmann P.** 2014. Impact of the isolation medium for detection of carbapenemase- producing *Enterobacteriaceae* using an updated version of the Carba NP. J. Med. Microbiol. **63:**772-776.
23. **Carvalhaes CG, Picão RC, Nicoletti AG, Xavier DE, Gales AC.** 2010. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. J. Antimicrob. Chemother. **65:**249-251.
24. **Cooper GL, Louie A, Baltch AL, Chu RC, Smith RP, Ritz WJ, Michelsen P.** 1993. Influence of zinc on *Pseudomonas aeruginosa* susceptibilities to imipenem. J. Clin. Microbiol. **31:**2366-2370.
25. **Schnaitman CA.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. **108:**545-552.
26. **Shang ES, Summers TA, Haake DA.** 1996. Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect. Immun. **64:**2322-30.
27. **Crago AM, Koronakis V.** 1998. *Salmonella* InvG forms a ring-like multimer that requires the InvH lipoprotein for outer membrane localization. Mol. Microbiol. **30:**47-56.
28. **Jones N**. 1999. Surfactants in membrane solubilisation. Int. J. Pharm. **177:**137-159.
29. **Pasteran F, Veliz O, Rapoport M, Guerriero L, Corso A.** 2011. Sensitive and specific modified Hodge test for KPC and metallo-β-lactamase detection in

474 *Pseudomonas aeruginosa* by use of a novel indicator strain, *Klebsiella*
475 *pneumoniae* ATCC 700603. J. Clin. Microbiol. **49:**4301-4303.

476 30. **Table 1.** Detection of clinical isolates producing NDM-1 carbapenemase using

477 the Modified Hodge Test (MHT) and the Triton Hodge Test (THT).

Species included (No. of isolates)	Isolate	Acquired ß-lactamase	MIC (µg/ml)			Assay result:			
						MHT		THT	
			IMP	MEM	ERT	MEM	ERT	MEM	ERT
A. baumannii (3)	17042	NDM-1	≥16	≥16	ND	-	-	+	+
	17232	NDM-1	≥16	≥16	ND	-	+ (weak) [a]	+	+
	17575	NDM-1	≥16	≥16	ND	+ (weak)	+	+	+
A. pittii (2)	15274	NDM-1	≥16	≥16	≥2	-	-	+	+
	15373	NDM-1	≥16	≥16	≥2	-	-	+	+
C. amanolaticus (1)	19108	NDM-1	≥16	8	≥2	-	-	+	+
C. braakii (1)	19329	NDM-1	≥16	≥16	≥2	-	-	+	+
C. freundii (3)	15375	NDM-1 + CTX-M-15	8	2	≥2	-	+ (weak)	+	+
	17571	NDM-1 + CTX-M-15	2	8	≥2	+ (weak)	+	+	+
	17572	NDM-1	8	≥16	≥2	+ (weak)	+	+	+
E. aerogenes (1)	17568	NDM-1	8	8	≥2	-	+	+	+
E. cloacae (3)	17464	NDM-1	≥16	≥16	≥2	+ (weak)	+ (weak)	+	+
	17581	NDM-1 + PER-2	≥16	≥16	≥2	-	-	+	+
	19074	NDM-1 + CTX-M-2	≥16	8	≥2	-	-	+	+
E. coli (6)	15792	NDM-1 + CTX-M-15	2	4	≥2	+	+	+	+
	17386	NDM-1	≥16	≥16	≥2	-	-	+	+
	17574	NDM-1 + CMY-2	2	≥16	≥2	+ (weak)	+ (weak)	+	+
	17758	NDM-1	≥16	≥16	≥2	-	-	+	+
	19269	NDM-1	4	≥16	≥2	-	-	+	+
	19426	NDM-1	8	8	≥2	-	-	+	+
K. pneumoniae (6)	13717	NDM-1 + CTX-M-15	4	4	≥2	-	-	+	+
	17047	NDM-1 + CTX-M-2	≥16	≥16	≥2	-	+ (weak)	+	+
	17277	NDM-1 + ESBL [b]	≥16	≥16	≥2	-	+ (weak)	+ (weak)	+
	17579	NDM-1 + PER-2	≥16	≥16	≥2	+ (weak)	+ (weak)	+	+
	17619	NDM-1 + CTX-M-15	4	≥16	≥2	-	-	+	+
	17624	NDM-1 + CTX-M-15	4	≥16	≥2	-	-	+	+
M. morganii (2)	17569	NDM-1	4	8	1	-	-	+	+
	17570	NDM-1	8	8	1	-	-	+	+
P. rettgeri (7)	15758	NDM-1	8	8	≥2	-	-	-	+ (weak)
	15973	NDM-1 + PER-2	≥16	16	≥2	-	-	+	+
	17154	NDM-1	≥16	8	≥2	-	-	+	+
	17156	NDM-1	≥16	≥16	≥2	-	-	+	+
	17159	NDM-1	≥16	8	≥2	-	-	+	+
	17560	NDM-1	≥16	≥16	≥2	-	-	+	+
	17561	NDM-1 + PER-2	≥16	≥16	1	-	-	+	+
P. stuartii (4)	17600	NDM-1	8	4	0.5	-	-	-	+ (weak)
	17617	NDM-1	≥16	2	1	-	-	+	+

	17638	NDM-1	8	2	≤0.5	+ (weak)	+ (weak)	+	+
	17687	NDM-1 + PER-2	≥16	≥16	≥2	-	-	-	+ (weak)
S. marcescens (1)	17468	NDM-1	8	8	≥2	-	-	+	+
No. positive/total (%)						**8/40 (20%)**	**13/40 (32.5%)**	**37/40 (92.5%)**	**40/40 (100%)**

Abbreviations: IMP, imipenem; MEM, meropenem; ERT, ertapenem; ND, not determined; +, positive; -, negative; ESBL, extended-spectrum ß-lactamase

[a] A size of enhanced growth of indicator strain less or equal than 3 mm was categorized as a weak positive result for the indicated assay.

[b] strain with synergism between disks of aztreonam and amoxicillin/clavulanate (phenotypic test indicating ESBL production) but negative PCRs targeted for usual ESBL genes.

487 **Table 2.** Results of the Modified Hodge Tests (MHT) and Triton Hodge Test (THT)

488 and carbapenem susceptibility of selected carbapenemase producers and nonproducers

489 clinical isolates.

Group (No. of isolates)	β-lactamase (No. of isolates)	Bacterial species included (No. of isolates)	% of resistance to (% of non-susceptibility to)[a]			No. (%) positive by			
						Modified Hodge Test (MHT)		Triton Hodge Test (THT)	
			IPM	MEM	ETP	MEM	ETP	MEM	ETP
MBLs (60)	***Enterobacteriaceae* (33):**								
	IMP-8 (15)	*C. freundii* (1), *E. coli* (2), *Enterobacter asburiae* (1), *E. cloacae* (7), *K. oxytoca* (1), *K. pneumoniae* (2), *S. marcescens* (1)	27% (75%)	27% (75%)	33% (87.5%)	15 (100%)	15 (100%)	15 (100%)	15 (100%)
	VIM-1 (1)	*E. coli* (1)	100%	100%	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	VIM-2 (16)	*E. cloacae* (6), *K. pneumoniae* (2), *P. rettgeri* (4), *P. stuartii* (4)	68.8% (81.3%)	81.3% (81.3%)	81.3% (100%)	16 (100%)	16 (100%)	16 (100%)	16 (100%)
	VIM-11 (1)	*E. cloacae* (1)	0% (0%)	0% (0%)	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	All *Enterobacteriaceae*					33 (100%)	33 (100%)	33 (100%)	33 (100%)
	Non-*Enterobacteriaceae* (27):								
	IMP-1 (4)	*Acinetobacter junii* (2), *Acinetobacter ursingii* (2)	100%	100%	ND	4 (100%)	4 (100%)	4 (100%)	4 (100%)
	IMP-13 (4)	*P. aeruginosa* (4)	50% (50%)	50% (50%)	ND	2 (50%) (uninterp.: 2)	2 (50%) (uninterp: 2)	3 (75%) (uninterp: 1)	3 (75%) (uninterp: 1)
	IMP-16 (4)	*P. aeruginosa* (4)	100%	100%	ND	2 (50%) (uninterp: 2)	2 (50%) (uninterp: 2)	4 (100%)	4 (100%)
	IMP-18 (1)	*P. aeruginosa* (1)	100%	100%	ND	0 (0%) (uninterp: 1)	0 (0%) (uninterp: 1)	1 (100%)	0 (0%) (uninterp: 1)
	VIM-2 (11)	*P. aeruginosa* (5), *Pseudomonas chlororaphis* (1), *Pseudomonas fulva* (1), *Pseudomonas monteilli* (1), *Pseudomonas oleovorans* (1), *Pseudomonas putida* (2)	100%	100%	ND	2 (18%) (uninterp: 5)[b]	5 (45%) (uninterp: 5)[b]	11 (100%)	11 (100%)
	VIM-11 (1)	*P. aeruginosa* (1)	100%	100%	ND	0 (0%) (uninterp: 1)	0[b](0%) (uninterp: 1)	1 (100%)	1 (100%)
	SPM-1 (2)	*P. aeruginosa* (2)	100%	100%	ND	2 (100%)	2 (100%)	2 (100%)	2 (100%)
	All non-*Enterobacteriaceae*					12 (44%)	15 (56%)	26 (96%)	25 (93%)
	***All MBLs* (60)**					**45 (75%)**	**48 (80%)**	**59 (98%)**	**58 (97%)**
Class A	***Enterobacteriaceae* (19):**								

carbapenemases (25)	KPC-2 (14)	*Citrobacter braakii* (1), *C. freundii* (1), *E. coli* (3), *E. cloacae* (2), *Leclercia adecarboxylata* (1), *K. oxytoca* (1), *K. pneumoniae* (5)	50% (85.7%)	50% (71.4%)	64.2% (85.7%)	14 (100%)	14 (100%)	14 (100%)	14 (100%)
	KPC-3 (1)	*K. pneumoniae* (1)	100%	100%	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	GES-3 (1)	*K. pneumoniae* (1)	100%	100%	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	NMC-A (1)	*E. cloacae* (1)	100%	100%	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	Sme-1b (2)	*S. marcescens* (2)	100%	50% (50%)	0% (0%)	1 (50%) (uninterp: 1)	1 (50%) (uninterp: 1)	2 (100%)	2 (100%)
	All *Enterobacteriaceae*					18 (95%)	18 (95%)	19 (100%)	19 (100%)
	Non-*Enterobacteriaceae* (6):								
	KPC-2 (5)	*P. aeruginosa* (5)	100%	100%	ND	5 (100%)	5 (100%)	5 (100%)	5 (100%)
	GES-5 (1)	*P. aeruginosa* (1)	100%	100%	ND	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	All non-*Enterobacteriaceae*					6 (100%)	6 (100%)	6 (100%)	6 (100%)
	***All Class A carbapenemases* (25)**					**24 (96%)**	**24 (96%)**	**25 (100%)**	**25 (100%)**
Class D carbapenemases (20)	OXA-48 (5)	*E. coli* (3), *K. oxytoca* (1), *K. pneumoniae* (1)	20% (80%)	40% (100%)	80% (100%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)
	OXA-163 (11)	*C. freundii* (1), *E. cloacae* (3), *E. coli* (2), *K. pneumoniae* (3), *P. stuartii* (2)	18.1% (27.3%)	27.3% (27.3%)	100%	11 (100%)	11 (100%)	11 (100%)	11 (100%)
	OXA-181 (1)	*K. pneumoniae* (1)	100%	100%	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	OXA-247 (2)	*E. coli* (1), *K. pneumoniae* (1)	50% (100%)	50% (100%)	50% (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)
	OXA-438 (1)	*E. coli* (1)	100%	100%	100%	1 (100%)	1 (100%)	1 (100%)	1 (100%)
	***All Class D carbapenemases* (20)**					**20 (100%)**	**20 (100%)**	**20 (100%)**	**20 (100%)**
Carbapenemase non-producers (40)	ESBLs + porin loss (20)[c]	*E. aerogenes* (1), *E. cloacae* (2), *K. pneumoniae* (16), *S. marcescens* (1)	10% (50%)	85% (90%)	95% (100%)	1 (5%)	2 (10%)	2 (10%)	3 (15%)
	Overexpression of chromosomal AmpC + porin loss (18)	*E. aerogenes* (2), *E. cloacae* (14), *E. coli* (2)	61.1% (100%)	44.4% (88.9%)	100%	1 (6%)	1 (6%)	1 (6%)	2 (11%)
	CMY-2 (2)	*E. coli* (1), *K. pneumoniae* (1)	0% (0%)	0% (0%)	0% (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	All *non-producers* (40)					**2 (5%)**	**3 (7.5%)**	**3 (7.5%)**	**5 (12.5%)**

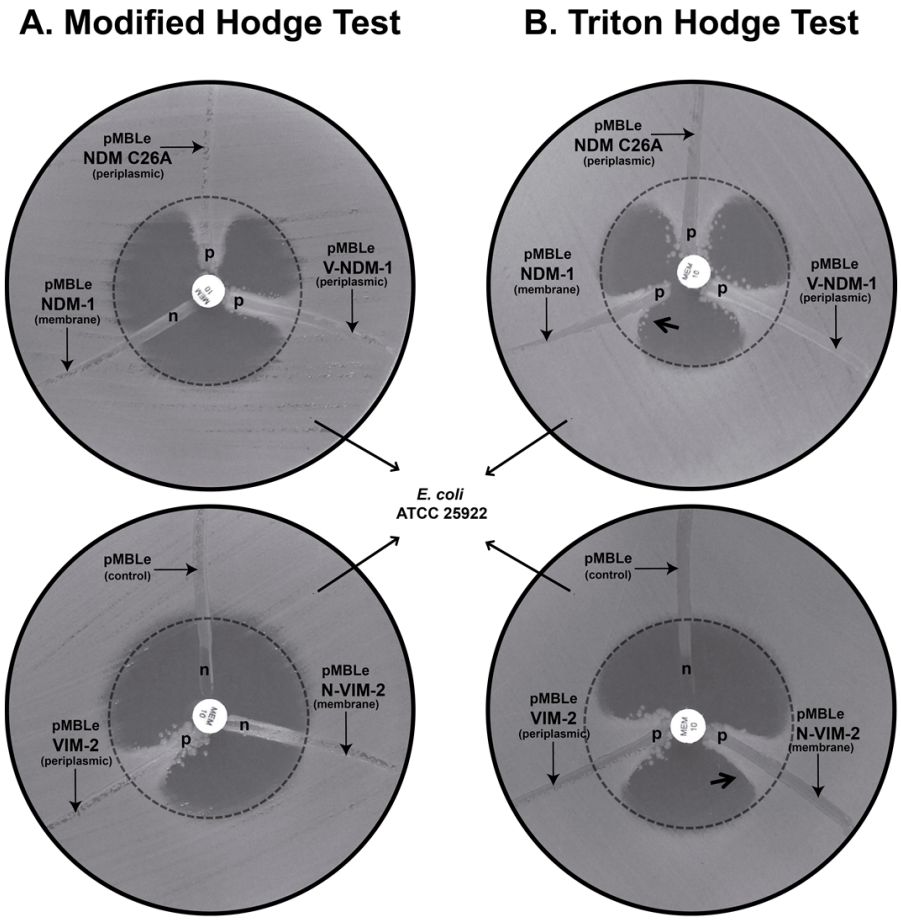
Abbreviations: IMI, imipenem; MEM, meropenem; ETP, ertapenem; ND, not determined; uninterp, uniterpretable test due to inhibition of growth of the indicator strain along the tested isolate.

[a] Intermediate plus resistant isolates to the indicated carbapenem (when applied).

[b] Four isolates of *P. aeruginosa* and one isolate of *P. putida* producing VIM-2 with uninterpretable results

[c] ESBLs included were (n): CTX-M-2 (16) and CTX-M-15 (4).

498 **Figure 1**



499

500 **Figure 1. Results of the Modified Hodge tests (MHT) and the Triton Hodge Test**
501 **(THT) for *Escherichia coli* DH5α strains harboring wild type and mutant variants**
502 **of NDM-1 and VIM-2 genes.** Meropenem (MEM) disk (10 µg) used as substrate.
503 Cellular localization (membrane or periplasmic) of MBLs is depicted between brackets.
504 The letter "p" indicates that MEM was hydrolyzed by the streaked cells; the letter "n"
505 indicates that MEM was not hydrolyzed by the streaked cells. [For more technical
506 details, readers may refer to CLSI (M100-S25, Table 3B and 3B-1, page 117) where the
507 MHT components and test interpretation is included].

Figure 2.

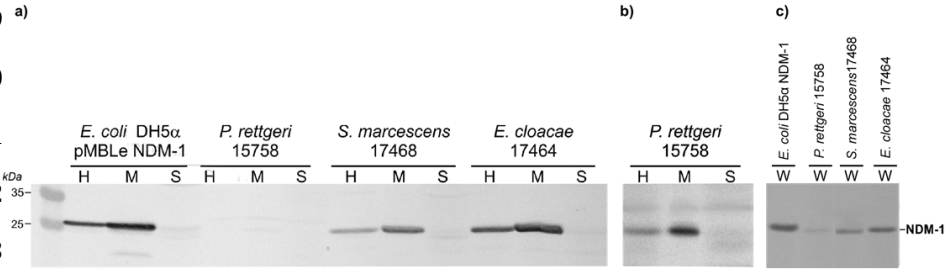
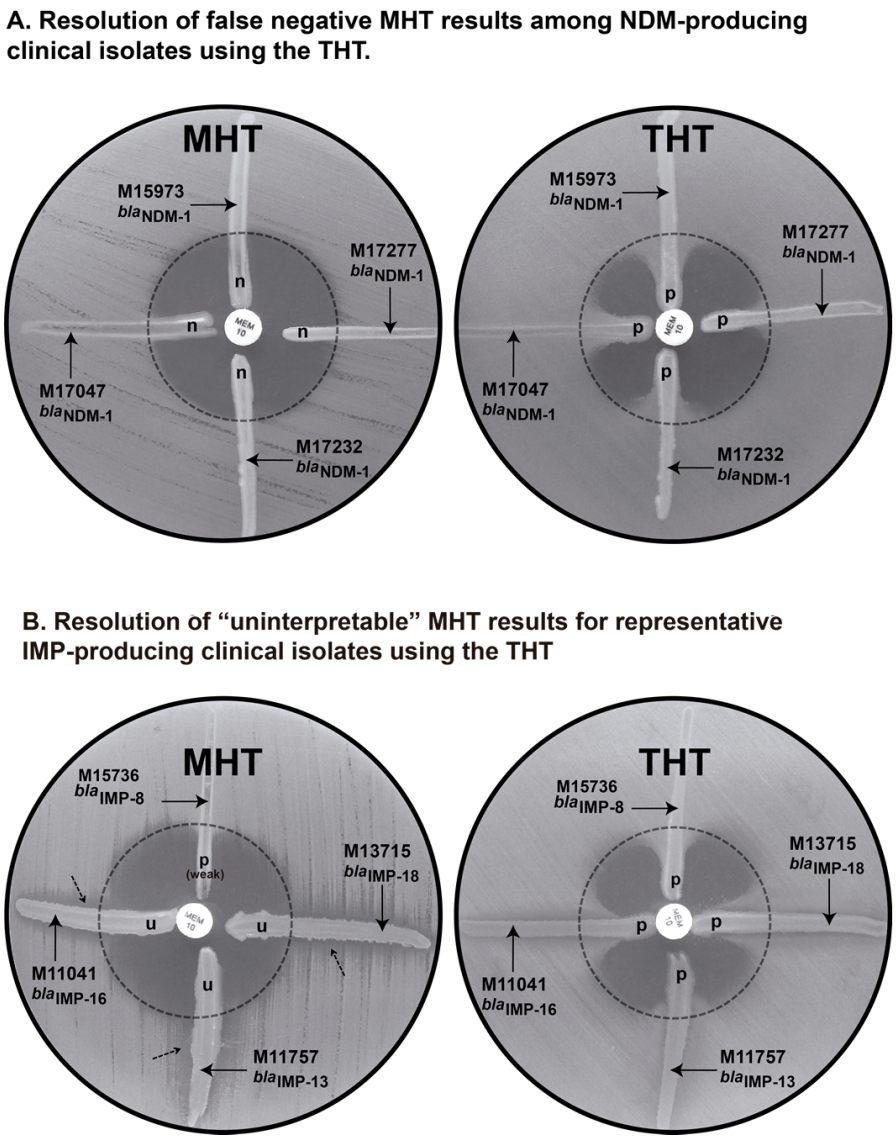


Figure 2. Membrane localization of NDM-1 in laboratory strain *E. coli* DH5α pMBLe NDM-1 and clinical strains *P. rettgeri* 15758, *S. marcesens* 17468 and *E. cloacae* 17464. Western blot detection of NDM-1 in membrane (M), soluble fractions (S) derived from bacterial homogenates (H) (using standard [2a] or an extended colorimetric reaction [from 5 to 20 minutes] of the secondary antibody [2b]) and whole cells (W) [2c].

521 **Figure 3**



522

523 **Figure 3. Comparative results of the Modified Hodge tests (MHT) versus the**
524 **Triton Hodge Test (THT) for representative carbapenemase-producing clinical**
525 **isolates.** Results obtained using a meropenem (MEM) disk (10 µg) as substrate. Strains
526 included: A) *P. rettgeri* M15973, *K. pneumoniae* M17047, *A. baumannii* M17232 and

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527 *K. pneumoniae* M17277. B) *E. cloacae* M15736, *P. aeruginosa* M11041, *P. aeruginosa*
528 M11757 and *P. aeruginosa* M13715. The letter "p" indicates that MEM was hydrolyzed
529 by the streaked cells; the letter "n" indicates that MEM was not hydrolyzed by the
530 streaked cells; the letter "u" indicates an uninterpretable result (the dotted arrow shows
531 inhibition of growth of the indicator strain *E. coli* ATCC 25922)