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Modification and evaluation of the Triton Hodge test for screening carbapenemase-producing *Enterobacteriaceae*

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ABSTRACT

Detection of carbapenemase-producing *Enterobacteriaceae* (CPE) has become critical for appropriate antimicrobial therapy and for controlling the spread of infection. We evaluated Triton Hodge test (THT) for screening CPE. A spreader can be used to apply more constant volume of Triton on whole surface of Mueller–Hinton agar (MHA), or alternatively, a 10-μL inoculating loop can be used to apply a 20% Triton solution lineally. The THT procedure can be simplified by eliminating the 1/10 dilution step of indicator bacteria from the McFarland 0.5 turbidity suspension. The presence of Triton in the MHA plates significantly increased the enhanced growth size of not only *Enterobacteriaceae* producing NDM-1–like enzymes but also those producing the most prevalent KPC-2–like enzyme, resulting in 100% sensitivity of the test.

1. Introduction

Carbapenemases have been increasingly detected in clinical isolates of *Enterobacteriaceae* in many countries. The detection of carbapenemase-producing *Enterobacteriaceae* (CPE) has become critical for appropriate antimicrobial therapy and for controlling the spread of infection (Lutgring and Limbago 2016). The detection of carbapenemase-producing Gram-negative bacteria in the laboratory has become more difficult due to the emergence of genetically novel and phenotypically diverse enzymes over the past 2 decades (Diene and Rolain 2014). Moreover, the challenges that laboratories face in different regions of the world significantly vary because of possible regional differences in the prevalent enzyme types (Logan and Weinstein 2017). Therefore, there is no single phenotypic test that is applicable in all situations (Tamma and Simner 2018).

We initially developed the modified Hodge test (MHT) to screen IMP-1– and VIM-2–producing species of *Pseudomonas* and *Acinetobacter*, which had emerged in South Korea (Lee et al. 2001). Anderson et al. (2007) evaluated the performance of MHT for identifying *Klebsiella pneumoniae* carbapenemase (KPC), which had emerged in the United States. Subsequently, the CLSI document M100-S19 2009 listed the test as a screening and confirmation test for KPC-producing *Enterobacteriaceae*. However, it has been shown that MHT has low sensitivity and specificity for carbapenemase detection due to increasing enzyme diversity. Van der Zwaluw et al. (2015) had developed the carbapenem inactivation method (CIM) to detect carbapenemase in Gram-negative bacilli. The CLSI document (2018) replaced MHT with the CarbaNP test (Nordmann et al. 2012) and the modified carbapenem inactivation method (mCIM) (Pierce et al. 2017).

The primary issue with MHT is very low sensitivity, particularly in detecting the increasing number of NDM-producing bacteria. Unlike all other known carbapenemases, NDM-1 is a lipoprotein anchored to the outer membrane of Gram-negative bacteria (Pasteran et al. 2016). It was Pasteran et al. (2016) who had verified NDM characteristics and reported that the Triton Hodge test (THT) could improve detection of not only isolates with NDM but also those with other carbapenemases. Performance of the THT was evaluated by Sun et al. (2017). THT may still be useful for some laboratories due to its simplicity and low cost.

The aims of this study were to develop a simpler THT method and evaluate the performance of a modified THT in detecting clinical *Enterobacteriaceae* isolates that are producing currently prevalent KPC- and NDM-type enzymes.

2. Materials and methods

2.1. Bacterial isolates

In the first stage of the study, we modified the THT and compared performance using stock strains carrying various carbapenemase

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genes. Isolates with carbapenemase types not reported in Korea were kindly provided by Professor D. M. Livermore, University of East Anglia, UK. In the second stage of the study, the test bacteria were clinical isolates of ertapenem-nonsusceptible *Enterobacteriaceae* isolated from July 2017 to June 2018 by the CPE surveillance team in a Korean university hospital. Once CPE was detected in a patient's clinical specimen, weekly stool specimens in addition to weekly original type of specimens were collected until CPE was not detected in 2 consecutive samples.

2.2. Species identification and carbapenemase gene detection

Species of the clinical isolates were identified using a Microflex MALDI-TOF mass spectrometer with Biotyper software 3.1 (Bruker Daltonics, Leipzig, Germany). Ertapenem susceptibility was determined using the VITEK 2 system AST N224 card (bioMerieux, Marcy-l'Étoile, France) and/or by the disk diffusion test using 10-μg ertapenem disks (Becton Dickinson, Sparks, MD).

PCR was performed to detect 5 different types of carbapenemase genes in ertapenem-nonsusceptible isolates. The oligonucleotide primers used for bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48} were those described by Ahn et al. (2016), and the primers used for bla_{NDM} were those described by Samuelsen et al. (2011). DNA was extracted by boiling for 10 min. After centrifugation at 13,000 rpm for 5 min, 1 μL of the supernatant was mixed with 19 μL of PreMix-Top (Bioneer, Daejeon, Korea). PCR was performed using the Veriti Thermal Cycler (Applied Biosystems, Foster City, CA). The condition was 5 min denaturation at 95 °C and 36 cycles of amplification consisting of 1 min at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with 7 min at 72 °C for the extension (YUMC Laboratory protocol). PCR amplicons were separated using Mupid-2plus instrument (Mupid Co., Tokyo, Japan) at 200 V for 20 min in 0.5× TAE with a 2% agarose gel (Safe shine green agarose gel, Biosesang, Seongnam, Korea).

2.3. Addition of Triton X-100 to Mueller–Hinton agar

Dehydrated Mueller–Hinton II agar (MHA, Becton-Dickinson) was used to prepare plates of MHA. Autoclave-sterilized medium was poured into 85-mm–diameter plates to obtain an approximate depth of 4 mm. Weekly batches of MHA plates were prepared.

We initially added 50 μL of Triton X-100 (Sigma-Aldrich, St Louis, MO) per MHA plate (0.2% vol/vol) as previously described by Pasteran et al. (2016). However, due to difficulties in pipetting viscous Triton at this concentration, we modified the protocol to transfer a 200-μL volume of 25% solution. The solution was spread over the entire surface by streaking with a cotton-tipped swab.

As an alternative, a cotton-tipped swab applicator or a 10-μL disposable plastic calibrated loop (SPL Life Sciences Co. Ltd., Pocheon City, Korea) was used to spread the Triton solution over an agar surface of approximately 1 × 4 cm from the center of the plate to the periphery ("linear application"). Prior to Triton transfer, 3 radial lines 120° apart were marked using a template of equilateral template on the back of an MHA agar plate (Fig. 1). For the cotton swab method, only the cotton portion was dipped into a test tube with a 25% or a 12.5% Triton solution. The solution was then spread linearly along the previously marked lines. Since the cotton swab method cannot deliver the same amount of Triton consistently, a 10-μL disposable plastic loop was also used. In this case, only the loop head was dipped vertically into a 20% Triton solution, a concentration which did not form a partial gel at room temperature. The Triton solution was then spread onto the MHA surface over an approximately 1 × 4-cm area. (See Fig. 2.)

By calculating the change in cotton swab weight, we estimated that the method delivered an average of 16 μL of 25% or 12.5% Triton solution, which corresponded to approximately 4 μL and 2 μL of 100% Triton, respectively, over an area comparable to the whole area application method. It was estimated that the 10-μL loop method resulted in the transfer of 2 μL of 100% Triton over an approximately 1 × 4-cm area.

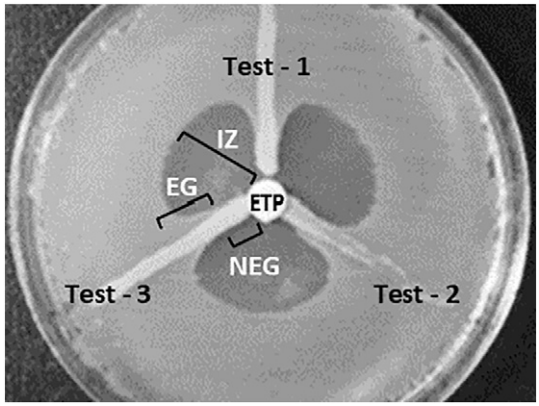

Fig. 1. Triton Hodge test. Three radial lines 120° apart were marked on the back of an MHA agar plate. In the Triton X-100 linear application, it was spread over the previously marked lines. Indicator bacteria were inoculated over the entire plate, and then test bacteria were inoculated linearly over the area with Triton X-100 applied. After incubating at 35 °C, the enhanced growth (EG) of indicator bacteria was determined by subtracting the size (mm) of nonenhanced growth (NEG) from the size (mm) of the inhibition zone (IZ), which was formed between test bacteria.

The Triton-applied plates were left at room temperature for approximately 30 min to allow the solution to absorb. The plates were then used for THT.

2.4. Modified Hodge test and Triton Hodge test

In the first stage of the study, frozen stock strains with known carbapenemase genes were subcultured and used to compare our modified THT methods (Table 1).

In the second stage of the study, ertapenem-nonsusceptible clinical isolates with no carbapenemase production information were obtained from the CPE surveillance team. These isolates were used to compare enhanced growth on a plain MHA plate and on an MHA plate with 10 μL of linearly applied 20% Triton (Table 2). To compare the effect of bacterial turbidity on enhanced growth, a McFarland 0.5 *Escherichia coli* ATCC 25922 suspension and a 1/10 dilution of the suspension were used to inoculate the agar surface with a cotton-tipped applicator. After approximately 30 min, the plate was inoculated with the test bacteria. A 10-μL disposable inoculating loop was used to lightly touch by the tip a confluent overnight bacterial culture and then streaked across the MHA surface by dragging the loop sideways from the center of the plate to the periphery, creating a narrow band of bacteria. For plates with linear application of Triton, the plate was inoculated along the previously marked line. A 10-μg ertapenem disk (Becton-Dickinson) was placed at the center of the plate and incubated at 35 °C for 16–18 h.

The Hodge test is a qualitative test, but in this study for comparison purpose, enhanced growth was measured with a ruler. Since enhanced growth is difficult to measure accurately, because of distortion of inhibition zone along the growth of test organism (Fig. 1), it was determined by subtracting size (mm) of nonenhanced growth (NEG) from the size (mm) of inhibition zone (IZ) which was formed in between test organisms.

Enhanced growth was arbitrarily defined: ≥4 mm was positive and ≤ 3 mm was negative. Our negative cutoff value (≤3 mm) was stricter than the value by Pasteran et al. (2016), who had defined this cutoff as weakly positive since they had observed that this cutoff level often caused discrepant values between two readers. In our study, enhanced growth was measured by two readers independently and blindly. When the differences were over 3 mm, measurements were repeated,

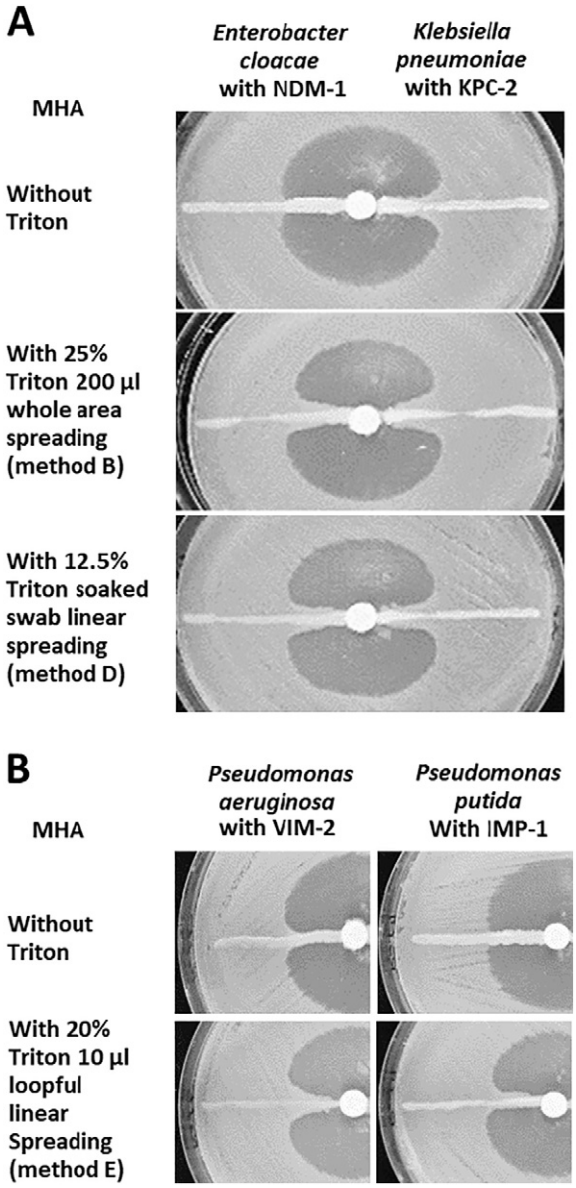

Fig. 2. The typical effect of Triton on enhanced growth on *Enterobacteriaceae* and glucose nonfermenting Gram-negative bacilli. (A) Enhanced growth was apparent only when Triton was added in the test of *Enterobacter cloacae* producing NDM-1, whereas enhanced growth was most apparent when 25% Triton was added in the test of *Klebsiella pneumoniae* producing KPC-2. (B) Enhanced growth was also apparent when 20% Triton was linearly spread using a 10- µL inoculating loop and *Pseudomonas aeruginosa* producing VIM-2 and *P. putida* producing IMP-1 were tested.

3. Results and discussion

3.1. Evaluation of modified THT

The first stage of the study was performed with stock strains carrying carbapenemase genes to compare Triton application methods. Among the isolates with class B enzyme-producing glucose-nonfermenting Gram-negative bacilli, 7 isolates (1 each of *Pseudomonas fluorescens*, *Pseudomonas putida,* and *Acinetobacter lwoffii* with IMP-1, 1 *A. lwoffii* with SIM-1, 1 each of *Pseudomonas aeruginosa* and *P. putida* with VIM-2, and 1 *P. aeruginosa* with VIM-3) were the MHT negative. However, all of these isolates were the THT positive (Table 1).

One isolate of *Acinetobacter pittii* producing NDM-1 showed enhanced growth of only 4 mm with MHA but showed 10–13 mm of enhanced growth with Triton X-100, depending on the application method (Table 1).

Among the class D carbapenemase-producing *Acinetobacter* spp., 2 isolates producing OXA-23 and the other 2 isolates producing OXA-27 and OXA-58 showed only 0–1 mm enhanced growth by MHT. However, all isolates showed greater enhanced growth with THT (Table 1).

Among the carbapenemase-producing *Enterobacteriaceae*, 1 isolate of *Klebsiella pneumoniae* with a class A KPC-2 enzyme showed greater enhanced growth on THT than on MHT (Table 1). Isolates of *Enterobacter cloacae* and *Citrobacter freundii* producing NDM-1 showed only 2 mm of enhanced growth on MHT, but the strains showed 6–10 mm and 4–10 mm of enhanced growth, respectively, with THT methods B and F. Enhanced growth on MHT and THT was small for isolates of *E. cloacae, E. coli,* and *K. pneumoniae* producing VIM-1 and 1 isolate each of 3 identical species with VIM-4 (Table 1). The poor performance of MHT and THT for detecting VIM-1 and VIM-4 producers could be explained by low carbapenem hydrolytic activity. VIM-1 and VIM-4 enzymes have been reported to have lower kcat/*Km* values against meropenem than VIM-2 (Lassaux et al. 2011).

One *E. coli* isolate producing OXA-48 showed greater enhanced growth on both MHT and THT, but 1 of the 2 *E. coli* isolates producing OXA-232 showed enhanced growth of only 3 mm on MHT compared to 7–9 mm on THT (Table 1). At the time of review by Mairi et al. (2018), there were 11 variants of OXA-48–like enzymes. In an evaluation of the CarbaNP test, Canadian isolates of *Enterobacteriaceae* were negative for 13 of 33 (39.4%) OXA-48–positive isolates and 3 of 6 OXA-181–positive isolates (Tijet et al. 2013). In a Korean evaluation of CIM, overall sensitivity and specificity were high, but the assay failed to detect 1 of the 5 *E. coli* isolates with OXA-232 (Song et al. 2016). In a study from Thailand, the mCIM test had shown high sensitivity, detecting all OXA-48–, OXA-181–, and OXA-232–producing isolates of *Enterobacteriaceae* (Laolerd et al. 2018).

In our present study, 1 *K. pneumoniae* isolate producing GES-5 showed no enhanced growth (0 mm) on MHT, and enhanced growth was only 0–2 mm with the Triton-added methods. The CarbaNP test was negative for all 3 GES-5-positive *Enterobacteriaceae* isolates (Tijet et al. 2013). The CIM test was negative for 3 of the 7 *K. pneumoniae* isolates producing GES-5 (Song et al. 2016). Among 31 GES β-lactamase variants, GES-2, -4, -5, -6, -14, and -18 have carbapenemase activity (Streling et al. 2018). Some carbapenemases, such as the GES- and OXA-48-types (Queenan and Bush 2007; Docquier et al. 2009), have relatively weak hydrolytic activity. Therefore, hydrolysis-based phenotypic methods could have lower detection rates, especially for GES-type (Tijet et al. 2013; Aguirre-Quiñonero et al. 2017) and some OXA-48-type variants, such as OXA-232 carbapenemases (Pierce et al. 2017).

When carbapenemase-producing stock strains were tested (Table 1), the overall median enhanced growth was larger (10 mm) by THT (method B) and smaller (4 mm) by MHT (method A). The median enhanced growth using methods C and D was similar, 9 mm and 8 mm, respectably. Methods E and F, with inoculum turbidity of McFarland 0.05 and 0.5, respectively, showed similar enhanced growth of 8 mm and 9 mm. The *P* values by the Wilcoxon paired-sample test for differences in enhance growth sizes according to method were A vs. B < 0.0001 (significant), B vs. C 0.161 (not significant), B vs. D 0.002 (significant), B vs. E 0.192 (not significant), and E vs. F 0.053 (not significant).

3.2. Comparative evaluation of MHT and THT

In the second stage of the study, clinical isolates of ertapenem-nonsusceptible *Enterobacteriaceae* with and without carbapenemase genes were obtained from the CPE screening team without carbapenemase production status.

The total number of ertapenem-nonsusceptible clinical isolates detected by the CPE surveillance team was 1072 from 350 patients and 9 hospital environments. The majority of patient specimens were sputum ($n = 128$), urine ($n = 92$), purulent discharges ($n = 74$), blood ($n = 56$), and stool ($n = 659$) (data not shown). Among the 21 species of

Table 1
Effect of Triton x-100 and turbidity of indicator organism on enhanced growth (mm) observed in ertapenem disk modified Hodge test of 43 stock strains with various carbapenemases

Species and carbapenemase		[A] MHA (0.05) [b]	Addition of Triton to MHA				
			Whole area spreading	Linear spreading to 1 × 4-cm area			
			[B] 25% [c] 200 μL (0.05)	[C] 25% soaked swab (0.05)	[D] 12.5% soaked swab (0.05)	[E] 20% 10 μL loopful [d] (0.05)	[F] 20% 10 μL loopful (0.5)
Glucose-nonfermenting Gram-negative bacilli							
Class B							
DIM-1	*P. aeruginosa* [a]	5	11	11	12	11	10
IMP-1	*P. aeruginosa* [a]	5	9	11	9	7	6
IMP-1	*P. fluorescens/P. putida*	0/0	12/7	12/8	12/7	12/5	10/5
IMP-1	*A. baumannii/A. junii/A. lwoffii*	5/7/2	12/13/8	12/11/8	12/12/6	13/14/10	12/13/6
IMP-1	*D. acidovorans*	11	12	10	10	6	11
IMP-4	*A. baumannii* [a]	8	12	12	12	15	13
IMP-6	*P. aeruginosa*	8	12	10	12	9	10
NDM-1	*A. pittii*	4	13	13	10	11	11
SIM-1	*A. baumannii/A. lwoffii*	6/3	13/12	12/12	10/12	16/14	13/14
VIM-1	*P. aeruginosa* [a]	0	2	1	0	1	2
VIM-2	*P. aeruginosa/P. putida/ P. monteilii*	0/2/4	10/7/10	9/10/8	8/7/6	8/6/4	9/5/7
VIM-2	*A. baumannii/A. junii/A. lwoffii*	6/4/9	10/13/10	9/12/10	11/12/8	7/13/14	8/13/12
VIM-3	*P. aeruginosa*	1	6	4	4	5	5
Class D							
OXA-23	*A. baumannii/A. baumannii*	2/1	8/8	9/5	8/7	6/8	9/7
OXA-25	*Acinetobacter* sp. [a]	4	11	12	9	12	11
OXA-26	*Acinetobacter* sp. [a]	7	11	10	9	14	11
OXA-27	*Acinetobacter* sp. [a]	1	10	7	9	10	10
OXA-58	*Acinetobacter* sp. [a]	0	10	7	7	10	9
Enterobacteriaceae							
Class A							
GES-5	*K. pneumoniae*	0	2	2	2	2	0
KPC-2	*K. pneumoniae*	6	11	12	9	11	10
Sme-1	*S. marcescens* [a]	9	10	9	10	8	8
Class B							
NDM-1	*E. cloacae/K. oxytoca/C. freundii*	2/4/2	8/9/9	10/9/10	6/7/4	7/6/9	7/9/9
VIM-1	*E. cloacae/ E. coli/K. pneumoniae* [a]	1/2/3	4/4/4	3/3/4	4/6/5	3/5/4	3/4/2
VIM-4	*E. cloacae/E. coli/K. pneumoniae*	2/4/4	5/5/7	4/3/9	5/4/7	5/4/6	4/3/5
VIM-19	*K. pneumoniae* [a]	7	11	13	12	12	11
Class D							
OXA-48	*E. coli*	9	14	9	12	10	10
OXA-232	*E. coli/E. coli*	3/5	7/9	9/10	8/7	9/8	8/8
All [e]	Range (mm)	0–11	2–14	1–13	0–12	1–16	0–14
	Median (mm)	4	10	9	8	8	9

[a] These strains were kindly provided by Professor D.M. Livermore, University of East Anglia, Norwich, UK.
[b] An overnight culture of the indicator bacterium, *E. coli* ATCC 25922, was visually adjusted to match a 0.5 McFarland standard (bioMerieux). A 0.05 turbidity suspension was obtained by making a 10-fold dilution of the 0.5 suspension.
[c] Triton was diluted with distilled water.
[d] A 10-μL plastic loop was used to transfer the Triton solution and spread it over an approximately 1 × 4-cm area between the center of the MHA plate to the periphery.
[e] *P* by Wilcoxon paired sample test: A vs. B (<0.0001), B vs. C (0.161), B vs. D (0.002), B vs. E (0.192), and E vs. F (0.053).

Enterobacteriaceae recovered, the majority of the isolates were *K. pneumoniae* (800, 71.2%), *E. coli* (189, 16.8%), and *Enterobacter* spp. (84, 7.5%) (data not shown).

In the second stage study, MHT refers to the original modified Hodge test using MHA (Lee et al. 2001), and THT refers to the method using MHA with a linear application of 10 μL of 20% Triton (Table 1, method F). This THT method was chosen from the results of the first stage study because it was simple to apply the Triton solution using an inoculating loop. In both methods, the turbidity of the indicator organism was adjusted to McFarland 0.5, not to a 1/10 dilution as used in the initial MHT (Tables 2, 3).

Among the ertapenem-nonsusceptible clinical isolates of *Enterobacteriaceae*, 444 were carbapenemase gene positive: 406 (91.4%) with bla_{KPC-2}-like, 29 (6.5%) with bla_{NDM-1}-like, and 9 (2.1%) with other carbapenemase genes.

The median enhanced growth values of the indicator bacteria by MHT and THT, respectively, were those with KPC-2–like, 6 mm and 11 mm, and those with NDM-1–like, 2 mm and 10 mm. As expected, the median enhanced growth values were greater for bacteria producing NDM-1–like enzymes in THT and moderately greater for bacteria producing KPC-2–like enzymes (Table 2). However, in isolates with OXA-48-like enzyme, the median enhanced growth in MHT and THT was 3 and 4 mm, respectively. The low median enhanced growth value in this group of bacteria using MHT was due to 1 isolate of *E. cloacae* producing VIM-2–like enzyme and 3 of the 5 isolates of *E. coli* and *K. pneumoniae* producing OXA-48–like enzymes. Two of the 3 isolates of *K. pneumoniae* producing OXA-48–like enzyme showed 3 mm of enhanced growth using THT.

The overall sensitivities of MHT and THT using ertapenem disks were 79.7% and 99.8%, respectively, based on the enhanced growth positive criteria (≥4 mm). The 99.8% sensitivity of THT was due to false-negative result in 2 of 3 isolates with OXA-181 (Table 2).

The respective sensitivities of MHT and THT by carbapenemase types were 83.9% and 100% for KPC-2–like, 31.0% and 100% for NDM-1–like, and 20.0% and 60.0% for OXA-48–like carbapenemases (Table 2).

In a study with 107 isolates of *Enterobacteriaceae* (Pasteran et al. 2016), the sensitivities of the MHT and THT using ertapenem disks were 31.2% and 100%, respectively, in detecting NDM, and 96% and 100%, respectively, in detecting class A carbapenemases. The sensitivities of both MHT and THT were 100% in detecting IMP- and VIM-type MBLs and OXA-48–like carbapenemases. In an evaluation of 6 phenotypic methods (Sun et al. 2017), the sensitivities of the CarbaNP test and CIM in detecting *Enterobacteriaceae* isolates with KPC-2 ($n = 18$), MBL ($n = 57$), and multiple carbapenemases ($n = 13$) were all 100%.

Table 2
Comparison of enhanced growth on MHA and on MHA with 20% Triton 10-μL linear spreading in ertapenem disk modified Hodge test of clinical *Enterobacteriaceae* isolates with carbapenemases.

Species (no. of isolates tested)	Enhanced growth [a]						*P* [c]
	MHA			MHA with 20% Triton 10-μL loopful linear spreading			
	Range (mm)	Median (mm)	No. (%) positive [b]	Range (mm)	Median (mm)	No. (%) positive [b]	
KPC-2–like							
K. pneumoniae (334)	1–14	8	276 (82.6)	4–16	10	334 (100)	
K. oxytoca (1)	4		1 (100)	10		1 (100)	
E. coli (55)	0–13	7	49 (89.1)	4–15	10	55 (100)	
C. freundii (8)	5–11	8	8 (100)	10–15	13	8 (100)	
C. braakii (1)	5		1 (100)	12		1 (100)	
C. koseri (1)	7		1 (100)	10		1 (100)	
E. aerogenes (5)	1–5	5	4 (80.0)	8–11	11	5 (100)	
E. kobei (1)	10		1 (100)	10		1 (100)	
All (406)	0–14	6	341 (83.9)	4–16	11	406 (100)	<0.0001
NDM-1–like							
E. coli (15)	0–10	5	4 (26.7)	6–15	11	15 (100)	
K. pneumoniae (8)	1–5	3	2 (25.0)	6–13	10	8 (100)	
E. cloacae (5)	2–5	4	3 (60.0)	6–10	8	5 (100)	
E. asburiae (1)	2		0 (0)	8		1 (100)	
All (29)	0–10	2	9 (31.0)	6–15	10	29 (100)	<0.0001
Others							
IMP-1–like							
E. cloacae (1)	8		1 (100)	10		1 (100)	
K. pneumoniae (1)	4		1 (100)	7		1 (100)	
VIM-2–like							
E. cloacae (1)	2		0 (0)	4		1 (100)	
All (3)	2–8	4	2 (66.7)	4–10	7	3 (100)	
OXA-48–like							
OXA-48 *E. coli* (1)	2		0 (0)	5		1 (100)	
OXA-181 *K. pneumoniae* (3)	2–3		0 (0)	3–4		1 (33.3)	
OXA-232 *K. ascorbata* (1)	7		1 (100)	9		1 (100)	
All (5)	2–7	3	1 (20.0)	3–9	4	3 (60.0)	
KPC-2- + IMP-1–like							
C. freundii (1)	12		1 (100)	13		1 (100)	
Total (444)	0–14	5	354 (79.7)	3–16	11	442 (99.5)	< 0,0001

[a] Turbidity of the indicator bacterium, *E. coli* ATCC 25922, was adjusted to McFarland 0.5.
[b] A positive result is arbitrarily defined as enhanced indicator bacterial growth ≥4 mm.
[c] *P* by Wilcoxon paired sample test.

It was interesting that, in their study, the sensitivity of THT using unspecified carbapenem disks was lower (91.3%) in testing 46 isolates with NDM-1 than the sensitivities of 100% using ertapenem disks both in our study and in the study of Pasteran et al. (2016).

In the second stage of the study, clinical isolates of ertapenem-nonsusceptible, carbapenemase gene-negative *Enterobacteriaceae* were used to determine the specificity of THT. Among the 134 carbapenemase gene-negative isolates, 2 (1.4%) of *K. pneumoniae* showed a false-positive MHT (≥4 mm of enhanced growth), whereas 12 (8.9%) isolates (6 isolates of *K. pneumoniae*, 5 *Enterobacter* spp., and 1 *E. coli*) showed a false-positive THT. The false-positive rate was significantly higher ($P < 0.0001$) using THT than using MHT (Table 3). In a

Table 3
Comparison of enhanced growth on MHA and on MHA with 20% Triton 10-μL linear spreading in ertapenem disk modified Hodge test of ertapenem-resistant, carbapenemase-negative clinical *Enterobacteriaceae* isolates.

Species (no. of isolates tested)	Enhanced growth on						*P* [b]
	MHA			MHA with 20% Triton 10-μL loopful linear spreading			
	Range (mm)	Median (mm)	No. (%) false positive [a]	Range (mm)	Median (mm)	No. (%) false positive [a]	
K. pneumoniae (88)	0–5	0	2 (2.2)	0–7	0	6 (6.8)	< 0,0001
E. coli (23)	0–1	0	0	0–7	0	1 (4.3)	0.008
E. aerogenes (5)	1	1	0	0–4	1.8	1 (20.0)	
E. cloacae (7)	1–3	0.85	0	0–4	2.0	2 (28.6)	
*E. **kobei** (2)*	0–1	0.5	0	1–2	1.5	0	
Enterobacter spp. (5)	0–1	0.6	0	1–5	2.6	2 (40.0)	
S. marcescens (2)	0–1	0.5	0	1–2	1.5	0	
H. alvei (1)	0		0	3		0	
P. rettgeri (1)	0		0	0		0	
Total (134)	0–5	0	2 (1.4)	0–7	1	12 (8.9)	< 0,0001

[a] A false positive is arbitrarily defined as enhanced indicator bacterial growth ≥4 mm.
[b] *P* by Wilcoxon paired sample test.

previous study, a false-positive MHT was related to a permeability defect along with AmpC overproduction in 3 of 4 *E. cloacae* isolates and with CTX-M-type ESBL-production in 1 of the 6 *K. pneumoniae* isolates (Girlich et al. 2012). In an mCIM evaluation, 1 of the 9 laboratories reported a false-positive result in an *E. coli* isolate producing TEM-52 (Pierce et al. 2017).

The CPE detection accuracy varies across phenotypic test. Local epidemiology of CPE genotypes, turnaround time, and ease of laboratory workflow integration should be considered when selecting a phenotypic assay for clinical use (Tamma et al. 2017). Several algorithms for phenotypic screening of CPE have been suggested. An ideal screening test was considered to be a test with 100% sensitivity and highest specificity, but with increasing carbapenemase diversity, it is hard to devise a screening test with such sensitivity and specificity (Robert et al. 2017). Because the Hodge test is a screening test, high sensitivity of THT can be considered more important than high specificity of MHT. In our study, sensitivities of MHT and THT were 79.7% and 99.5%, respectively (Table 2), and specificities were 98.6% and 91.1%, respectively (Table 3).

The sensitivity of a phenotypic carbapenemase detection method can be affected by the presence and proportion of bacteria with weak hydrolytic enzymes, such as GES and some OXA-48 variants. In our study, the sensitivity of THT was 100% for detection of *Enterobacteriaceae* producing KPC-2–like and NDM-1–like carbapenemase. The overall sensitivity of THT (99.5%) was much higher than that of MHT (79.7%) ($P < 0.0001$) due to the presence of only small number of isolates with OXA-48–like enzymes and the absence of GES-type enzyme (Table 2). The routine CPE detection algorithm at the hospital did not include PCR for the GES gene because, in a recent study, the gene was not detected among the 393 ertapenem-nonsusceptible *Enterobacteriaceae* obtained from 5 hospitals (Jeong et al. 2016). However, GES-5-producing *K. pneumoniae* were detected at a Korean hospital in 2004 (Jeong et al. 2005).

THT procedure is almost identical to that of MHT, but the sensitivity is much higher at least in detecting currently prevalent KPC-2 and NDM-1, and 4-h incubation step in mCIM is not required. Therefore, laboratories using mCIM may still use THT when they do not have time to process by mCIM. A spreader can be used to apply more constant volume (250 μL of 20%) Triton on whole surface of MHA, or alternatively, a 10-μL inoculating loop can be used to apply a 20% Triton solution lineally. THT is simple to perform but requires an additional step of adding Triton to the MHA plates. It has been reported that MHA plates with added Triton were stable for up to 12 weeks at 4 °C (Pasteran et al. 2016). In our study, the effect of linearly applied Triton to MHA plates was observed for at least 1 week at 4 °C (data not shown). We did not evaluate the effect of longer storage time because the plates lost moisture and because it was more practical to add Triton to the MHA plates as needed on the day of testing.

Limitations of our study were that the clinical isolates were from only 1 hospital, in Korea, and included only 3 isolates with IPM- and VIM-type MBL and 6 isolates with OXA-48–like enzyme, although isolates with KPC-2–like and NDM-1–like enzymes were large in number. Also, we did not compare performance of THT with that of currently recommended mCIM.

In conclusion, a spreader can be used to apply more constant volume of Triton on the whole surface of Mueller–Hinton agar (MHA), or alternatively, a 10-μL inoculating loop can be used to apply a 20% Triton solution lineally. The THT procedure can be simplified by eliminating the 1/10 dilution step of indicator bacteria from the McFarland 0.5 turbidity suspension. The presence of Triton in the MHA plates significantly increased the enhanced growth size of not only *Enterobacteriaceae* isolates with NDM-1–like enzymes but also those with the most prevalent KPC-2–like enzyme, resulting in 100%sensitivity of the test.

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