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A combined disc method with resazurin agar plate assay for early phenotypic screening of KPC, MBL and OXA-48 carbapenemases among Enterobacteriaceae

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Running title: Resazurin Plate Detects Carbapenemase

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Abstract

Aim: To validate a combined disc method along with resazurin chromogenic agar for early screening and differentiation of *Klebsiella pneumoniae* carbapenemase, metallo-β-lactamase and OXA-48 carbapenemase-producing Enterobacteriaceae.

Methods and Results: The combined disc test comprising of meropenem alone and with EDTA, phenylboronic acid, or both EDTA and phenylboronic acid, and temocillin alone were evaluated with the resazurin chromogenic agar plate assay against a total of 86 molecularly-confirmed Enterobacteriaceae clinical isolates (11 metallo-β-lactamases, 8 *Klebsiella pneumoniae* carbapenemases, 11 OXA-48, 32 AmpC and 15 extended-spectrum-β-lactamase producers and 9 co-producers of extended-spectrum-β-lactamase and AmpC). The inhibition zone diameters were measured and interpreted at seven hours for the presence of carbapenemase. All carbapenemase producers were phenotypically distinguished by this assay with 100% sensitivity and specificity.

Conclusions: This early phenotypic method is very simple, inexpensive, and reliable in the detection and differentiation of carbapenemase-producing Enterobacteriaceae. It could be exploited in any microbiological laboratory for diagnosis of these recalcitrant bacteria.

Significance and Impact of study: This assay poses excellent performance in discrimination of *Klebsiella pneumoniae* carbapenemase, metallo-β-lactamase and OXA-48 carbapenemases within seven hours, which is much faster than conventional disc diffusion methods. The rapid detection could help clinicians screen patients, control infection, and provide epidemiological surveillance.

Keywords: Carbapenemase, Enterobacteriaceae, combined disc, resazurin, phenotypic test.

Introduction

Resistance to carbapenems, a "last resort" β-lactam antibiotic for the treatment of Gram negative bacteria, in Enterobacteriaceae, is increasing at an alarming rate and becoming one of the most serious concerns in public-health worldwide (Tangden and Giske 2015). Carbapenemases have been recognised since imipenem was approved for clinical use in the 1980s (Walsh 2010). The first carbapenemase (NmcA) was identified in the clinical isolate of *Enterobacter cloacae* in 1993 and since then numerous carbapenemase-encoded genes in Enterobacteriaceae have been identified (Naas and Nordmann 1994). Three major classes of clinically-important carbapenemases have molecularly been classified; Ambler class A (mostly *Klebsiella pneumoniae* carbapenemase (KPC)), class B metallo-β-lactamase (MBL: IMP, VIM and NDM) and class D enzymes with carbapenemase activity (mostly OXA-48 and OXA-181) (Patel and Bonomo 2013). Currently, the USA, Israel, Greece and Italy are endemic for KPC, while OXA-48- producing *Kl. pneumoniae* and *Escherichia coli* have extensively been identified in North Africa and Turkey. The Indian subcontinent is an important reservoir of NDM-producing-Enterobacteriaceae, especially in *Kl. pneumoniae* and *E. coli*, as well as KPC and OXA-48 like-producing isolates (mostly OXA-181) (Nordmann and Poirel 2014).

Detection of carbapenemase production among Enterobacteriaceae and other Gram negative bacteria is more challenging as a result of multiple resistance mechanisms in the same strain (Nordmann *et al.* 2012). Several carbapenemase- identifying assays have been developed and can be grouped as phenotypic (based on synergistic effect between antibiotics and carbapenemase inhibitors), genotypic (PCR-based detection), biochemical-based methods (e.g. Carba NP test and Blue-carba test) (Dortet *et al.* 2015; Miriagou *et al.* 2013; Pires et al. 2013). Furthermore, MALDI-TOF and immunochromatography detection methods are also available (Vogne *et al*. 2014; Glupczynski *et al*. 2016). Earlier carbapenemase screening

methods relied upon the antimicrobial susceptibility profile determined by disc diffusion test and minimum inhibitory concentration (MIC) value from broth dilution method or by automated system (Miriagou *et al.* 2010). The cloverleaf test or the Modified Hodge Test (MHT) has been recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2009 as a confirmatory test for carbapenemase production in isolates demonstrating reduced susceptibility to carbapenem antibiotics. There are several shortcomings of these tests as they have poor specificity and sensitivity and relatively slow turnaround times (Carvalhaes *et al.* 2010).

Combined disc-inhibitor synergy tests have been used widely to discriminate different classes of carbapenemases. Boronic acids, particularly phenylbornonic acid (PBA), have been used to inhibit class A KPC activity, while metal chelating agents such as EDTA and dipicolinic acid (DPA) have been used to inhibit MBL activity (Giske *et al.* 2011; Tsakris *et al.* 2011; Nordmann *et al.* 2012). The temocillin (TEM) resistance profile (MIC• 128 µg ml^{-1} or zone diameter of 30 µg disc ≤ 10 mm) has been suggested as a phenotypic marker of OXA-48-producing Gram negative bacteria where there is a decrease in carbapenem susceptibility and absence of synergistic effect of carbapenem plus KPC or MBL inhibitors (Hartl *et al.* 2013; van Dijk *et al.* 2014; Woodford *et al.* 2014). A time to result of a disc diffusion method usually takes at least 18 h or overnight. The colorimetric plate containing resazurin showed excellent performance and reproducibility for disc diffusion susceptibility testing in *E. coli* isolates (Sener *et al.* 2011). The resazurin reduction assay is a colorimetric method that has extensively been used as an indicator for cell growth, cell viability, toxicity and indirect antimicrobial susceptibility testing. This dye is non-toxic to cells and stable in culture media. A blue coloured resazurin is irreversibly converted to a pink coloured resorufin by active cells (O'Brien et al. 2000; Sarker et al. 2007). No studies have been reported on phenotypic detection for β-lactamases using this colorimetric assay. This study describes the resazurin

chromogenic agar (RCA) plate along with combined disc-inhibitor synergy test for early screening and differentiation of KPC, MBL and OXA-48 carbapenemases.

Materials and Methods

Bacterial isolates and RCA plate preparation

A sum of 86 β-lactamase-producing Enterobacteriaceae UK clinical isolates (collected between 2012-2015), including 11 MBL producers, (5 NDM-1s, 1 NDM, 1 IMP-1 and 1 VIM-1+SHV-102, 1 VIM-1+SHV-12 and 2 VIM-types) 8 KPC producers (2 KPC-2s, 4 KPC-3s, 1 KPC-4 and 1 KPC-type), 11 OXA-48 producers and 56 non-carbapenemase-producing strains (32 AmpCs, 15 ESBLs and 9 co-producers of ESBL and AmpC), were used to validate RCA plate assay in the present study. The bacterial strains employed were *E. coli* (n=25), *Kl. pneumonia*e (n=28), *Ent. aerogenes* (n=12), *Ent. cloacae* (n=13), *Morganella morganii* (n=2), *Citrobacter freundii* (n=4), *Klebsiella oxytoca* (n=1) and *Klebsiella ozaenae* (n=1) (Table 1. Supplementary data). Non-β-lactamase-producing *E. coli* ATCC 25922 was used as a negative control strain. The isolates were biochemically and molecularly identified by PCR and sequencing following previous reports (Ellington *et al.* 2007; Dallenne *et al.* 2010; Poirel *et al.* 2011).

The RCA plates were prepared according to Sener and colleagues (Sener *et al.* 2011). Briefly, 25 mg of resazurin sodium salt (Sigma-Aldrich) was dissolved in 10 ml of sterile water and sterilised by filtration through 0.2 µm syringe filter. The sterile resazurin solution was added to 990 ml sterile Mueller-Hinton (MH) agar (Oxoid, UK) when the temperature of the medium reached approximately 45-50 ºC (to a final concentration of 25 µg ml^{-1}). The resazurin-containing MH agar was gently mixed prior to pouring 25 ml of the solution or approximately 4 mm depth into 90 mm circular petri dishes. Uninoculated RCA plates were stored in the fridge (4 ºC) for up to a week and kept away from the light.

Disc preparation and experiment procedure

Meropenem (MER) discs (10 µg) were prepared by supplementing blank discs (6.5 mm, MAST Diagnostic Group, UK) with 10 µl of 1 mg ml^{-1} MER (Sigma-Aldrich). Dried MER discs were then impregnated with 10 µl of PBA (Sigma-Aldrich) at a concentration of 40 mg ml^{-1} and EDTA at 75 mg ml^{-1} to obtain final amount of 400 µg/disc and 750 µg/disc, respectively. PBA was dissolved in dimethylsulphoxide (Sigma-Aldrich) and sterile water as previously recommended (Coudron 2005). EDTA and MER were dissolved in sterile water. Discs were air-dried in the cabinet for one hour prior to use.

The disc susceptibility testing was performed in accordance with the CLSI guideline (Clinical Laboratory Standards Institute 2010). A sterile swab was dipped in a 0.5 McFarland standard suspension of test bacteria and spread thoroughly on entire RCA's surface. Five discs including MER, MER+PBA, MER+EDTA, MER+PBA+EDTA and 30 µg TEM (MAST Diagnostic Group, UK) were firmly placed at equidistant points on the surface of the RCA plate. Following incubation at 37 ºC for seven hours, the diameters of the blue zones of inhibition were measured. A change in the colour of medium from blue (resazurin) to pink (resorufin) was visually observed in live bacteria. No colorimetric change was indicative of bacterial growth inhibition. The results were interpreted according to previously described assays with additional modifications (Table 2) (Miriagou *et al.* 2013; van Dijk *et al.* 2014). Synergy between MER and EDTA and PBA was considered as positive results for MBL and KPC, respectively. Absence of synergy between MER and EDTA or PBA with TEM zone diameter (≤ 10 mm) was denoted a positive result for OXA-48-producing isolates (Table 2). Sensitivity and specificity values were calculated by comparing results from RCA assay to molecular characterisation results. SPSS statistical analysis software was used to analyse the data and create box-and-whisker plot.

Results

The colorimetric phenotypic method using RCA plate with combined disc test for early detection and differentiation of MBL, KPC and OXA-48 carbapenemases explicitly demonstrated zone diameters within seven hours (Fig. 2). Distribution of the zone diameters of MER with and without PBA or EDTA and TEM alone against carbapenemase and non-carbapenemase-producing Enterobacteriaceae are shown in Fig.1. In MBL producers, the range of zone diameters of MER and MER+PBA was 6.5-20 mm and 6.5-19 mm, respectively. The median diameters for these discs were equally 17 mm. Discs containing EDTA (median = 26 mm and range = 23-27 mm) resulted in increased zone diameters compared with the discs without EDTA. The range of TEM zone diameters varied from 6.5-17 mm (median = 6.5 mm) as shown in Fig. 1A. The combined disc test using MER and EDTA successfully detected all MBL producers without false positive results in non-MBL isolates (sensitivity 100%, specificity 100%; Table 2).

For detection of KPC-producing Enterobacteriaceae, an increase in zone diameters was observed in MER+PBA (median = 22 mm and range = 19-27 mm) and MER+PBA+EDTA (median = 22.5 mm and range = 19-27 mm) compared with MER alone (median = 15 mm and range = 8-20 mm) or MER+EDTA (median = 16mm and range = 7-21mm). Synergistic effects of PBA was found only in KPC strains. The median zone diameter of TEM was 14 mm (range = 11-20 mm) as shown in Fig. 1B. The sensitivity and specificity values of PBA synergy test along with RCA assay for detection of KPC-producing Enterobacteriaceae were 100% (Table 2).

No difference in zone diameters of MER disc alone (median = 21 mm and range = 8-25 mm) and with PBA (median = 21 mm and range = 11-26 mm), EDTA (median = 21 mm and range = 12-25 mm), or PBA+EDTA (median = 22 mm and range = 12-25 mm) was observed in OXA-48 producers. These findings indicated no synergistic effect between PBA or EDTA

and MER. TEM inhibition zone diameters were ≤ 10mm (median = 6.5 mm and range = 6.5-9 mm) for all tested OXA-48 producers (Fig.1C). The zone diameters of TEM considered together with an absent synergistic effect of MER and PBA or EDTA were good indicators for identification of OXA-48-producing isolates (sensitivity 100% and specificity 100%; Table 2). In non-carbapenemase producers, there was no significant difference in zone diameters between MER alone and PBA or EDTA. The median diameter for TEM was 19 mm (range = 13-26) against these isolates (Fig. 1D). In addition, 10 µg MER clearly inhibited the growth of reference strain *E. coli* ATCC 25992. The zone diameter against this strain was 29 mm, which was in the quality control range (28- 34 mm) for non-fastidious organisms in CLSI document (Clinical Laboratory Standards Institute 2014). The results in the present investigation using combined disc method with RCA clearly discriminated different types of carbapenemases without discrepancy.

Discussion

Carbapenem resistance in Enterobacteriaceae poses a challenging issue for treatment and infection control. The rapid diagnostic test plays an important role in guiding clinicians to appropriate antibiotic administration and minimising treatment failure (Nordmann *et al.* 2012). Molecular-based methods for characterisation of carbapenemase-producing Enterobacteriaceae are restrictive due to high cost, requirement of skilled and experienced tecnicians and more importantly the inability to detect novel carbapenemase-encoding genes (Picao *et al.* 2008). Current combined disc synergy tests with β-lactamase inhibitors are simple, inexpensive and able to discriminate the different types of carbapenemases effectively, but they are limited in time as results require at least 18 h incubation (Osei Sekyere *et al.* 2015). To improve turnaround time, in the present study we conducted RCA

plate along with combined disc method for early screening of clinically-important carbapenemases including MBL, KPC and OXA-48.

For detection of MBL-producing Enterobacteriaceae, EDTA synergy test was able to detect all test MBL producers with 100% sensitivity and specificity. This result agrees with a study previously reported by Tsakris *et al.* (2010). They found that the combined disc method containing EDTA successfully detected all clinical isolates of VIM-producing Enterobacteriaceae. Suprisingly, some studies reported using EDTA as a MBL inhibitor and MER as a substrate gave some false-positive results in non-MBL-producing *Kl. pneumoniae* (Giske *et al.* 2011). Similarly, a combined disc method supplemented with imipenem and DPA showed better activity than EDTA against *Pseudomonas* spp. and *Acinetobacter* spp. producing IMP-1-like, VIM-2-like, and SIM-1-type MBLs (Yong *et al.* 2012). Giske *et al.* (2011) found that DPA synergy test with MER as a substrate had 100% in both sensitivity and specificity, which had superior performance in detection of MBL producers compared with EDTA. A good sensitivity (90%) and specificity (96%) of DPA synergy test was also previously reported by van Dijk *et al.* (2014) for detection of MBL-producing Enterobacteriaceae. Nevertheless, a study performed by Pitout *et al.* (2005) found that MER was more effective than imipenem and suggested the use of MER in combination with EDTA for detection of MBL-producing *Pseudomonas aeruginosa*.

The result of PBA synergy test in the present study using 400 µg PBA was reliably able to detect all KPC-producing Enterobacteriaceae clinical isolates (100% sensitivity). Tsakris *et al.* (2011) reported a few false-positive results were observed (97.6% specificity) and also PBA was more effective than aminophenylboronic acid in detection of KPC-producing isolates. A similar sensitivity and specificity from PBA synergy test for detecting KPC-producing Enterobacteriaceae was also reported by several studies (Tsakris *et al.* 2010; van Dijk *et al.* 2014). PBA synergy test was not only positive in KPC producers, but it was also

observed in producers of AmpC plus porin loss. It has been suggested that using cloxacillin synergy test can potentially discriminate between KPC and AmpC plus porin loss (Giske *et al.* 2011).

TEM zone diameter ≤10 mm considered together with absence in synergy of MER and EDTA or PBA was able to detect all OXA-48-producing Enterobacteriaceae. The results from the present study were consistent with several studies reporting excellent sensitivity and specificity of TEM disc in detection of OXA-48-like enzymes (OXA-48, OXA-162, OXA-181 and OXA-204) (Hartl *et al.* 2013; van Dijk *et al.* 2014; Oueslati *et al.* 2015). The zone diameters of MER discs against all non-carbapenemase-producing Enterobacteriaceae including ESBL and AmpC isolates were in the susceptible range in CLSI guideline (Clinical Laboratory Standards Institute 2014).

In conclusion, the combined disc method is effectively applicable in any microbiological laboratory. Using this test along with RCA assay is very simple and provides a faster result compared with the combined disc method alone. The result from RCA assay is visually easy to interpret. It also demonstrates excellent sensitivity and specificity for differentiation of MBL, KPC and OXA-48-producing Enterobacteriaceae. The RCA assay can be applied to commercially available discs, such as MAST-CDS (Mast Group, UK) and Rosco Diagnostica A/S (Denmark). However, further studies should be performed against a larger sample size of clinical isolates with co-producers of class A and class B or AmpC plus porin loss to establish the robustness of this assay. The early detection of carbapenemase would aid healthcare professionals to manage patients, control the spread of infection and for epidemiological surveillance purpose.

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Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Carbapenemase-producing-and non-carbapenemase-producing isolates used in this study

Group of β-lactamases	Molecular types	Species (no. of strains)
Carbapenemase producers		
Ambler class A KPC (n =8)	KPC-2	*Kl. pneumoniae* (1)
		Kl. oxytoca (1)
	KPC-3	*Kl. pneumoniae* (3)
		E. coli (1)
	KPC-4	*E. coli* (1)
	KPC-type	*Kl. pneumoniae* (1)
Ambler class B MBL (n=11)	IMP-1	*Kl. ozaenae* (1)
	NDM-1	*Ent. cloacae* (1)
		Kl. pneumoniae (3)
		E.coli (1)
	NDM-type	*Ent. cloacae* (1)
	VIM-1	*Kl. pneumoniae* (2)
	VIM-1+SHV-12	*Kl. pneumoniae* (2)
Ambler class D OXA-48 like ((n=11)	OXA-48	*Ent. cloacae* (2)
		Kl. pneumoniae (5)
		E.coli (4)
Non-carbapenemase producers		
Ambler class A ESBL (n=15)	TEM-214	*E.coli* (1)
	TEM-71+SHV-27	*Kl. pneumoniae* (1)
	TEM-10	*E.coli* (1)
	TEM-115+SHV-27	*Kl. pneumoniae* (1)
	TEM-type	*E.coli* (1)
		Kl. pneumoniae (1)
	TEM-53+SHV-27	*Kl. pneumoniae* (1)
	SHV-27	*Kl. pneumoniae* (1)
	SHV-18	*Kl. pneumoniae* (1)
	CTX-M+SHV-type	*Kl. pneumoniae* (1)
	CTX-M-15	*E.coli* (1)
	CTX-M-15+SHV-27	*Kl. pneumoniae* (1)
	CTX-M-3	*E.coli* (2)
	CTX-M-type	*E.coli* (1)
	CTX-M+SHV+TEM	
Ambler class C AmpC (n=32)	DHA-1	*Kl. pneumoniae* (1)
		E. coli (1)
	CMY-2	*E. coli* (2)
	CMY-112	*Cit. freundii* (1)
	ACT-31	*Ent. aerogenes* (1)
	ACT-32	*Ent. cloacae* (3)
	FOX-3	*E. coli* (1)
	MOX-1 MOX-2 CMY-1 CMY-8 to CMY-11	*Cit. freundii* (1)
		E. coli (1)
	MIR-1T ACT-1	*Ent. cloacae* (2)
		Ent. aerogenes (2)
		Kl. pneumoniae (1)
		E. coli (1)
	FOX-1 TO FOX5b	*Ent. aerogenes* (2)
		Ent. cloacae (2)
	LAT-1 TO LAT-4,CMY-2 TO CMY-7 BIL-1	*E. coli* (1)
	Depressed AmpC	*E. coli* (2)
		Ent. cloacae (1)
		Morganella morganii (1)
	Inducible AmpC	*Ent. aerogenes* (1)
	DHA-1 DHA-2	*Ent. aerogenes* (1)
		E. coli (1)
		Kl. pneumoniae (1)
		M. morganii (1)
Class A + Class C (n=9)	TEM+ CTX-M + MIR-1T ACT-1	*Ent. aerogenes* (1)
	SHV+CTX-M-9 + MIR-1 ACT-1	*Ent. aerogenes* (1)
	ESBL + ACT-32	*Ent. cloacae* (1)
	CTX-M-3+AmpC	*Cit. freundii* (2)

SHV + MIR-1T ACT-1	*Ent. aerogenes* (1)
TEM+SHV+ MIR-1T ACT-1	*Ent. aerogenes* (1)
CTX-M-9+AmpC	*E. coli* (1)
SHV-12+CTX-M-9+ACT-32	*Ent. aerogenes* (1)

Table 2. Interpretation criteria for combined disc synergy test and Performance of combined disc test along with RCA assay for the early detection of carbapenemase-producing Enterobacteriaceae.

Test	MBL	KPC	OXA-48	Sensitivity	Specificity
B-A and D-C (EDTA synergy) • 5 mm	+	-	-	100 (11/11)	100 (76/76)
C-A and D-B (PBA synergy) • 5 mm	-	+	-	100 (8/8)	100 (79/79)
E ≤ 10 mm*	-	-	+	100 (11/11)	100 (76/76)

A = meropenem disc; B = meropenem + EDTA disc; C =meropenem + phenylboronic acid (PBA) disc; D = meropenem + EDTA + PBA disc; E=temocillin disc

* Considered when absence of EDTA, PBA, and EDTA+PBA synergy tests.

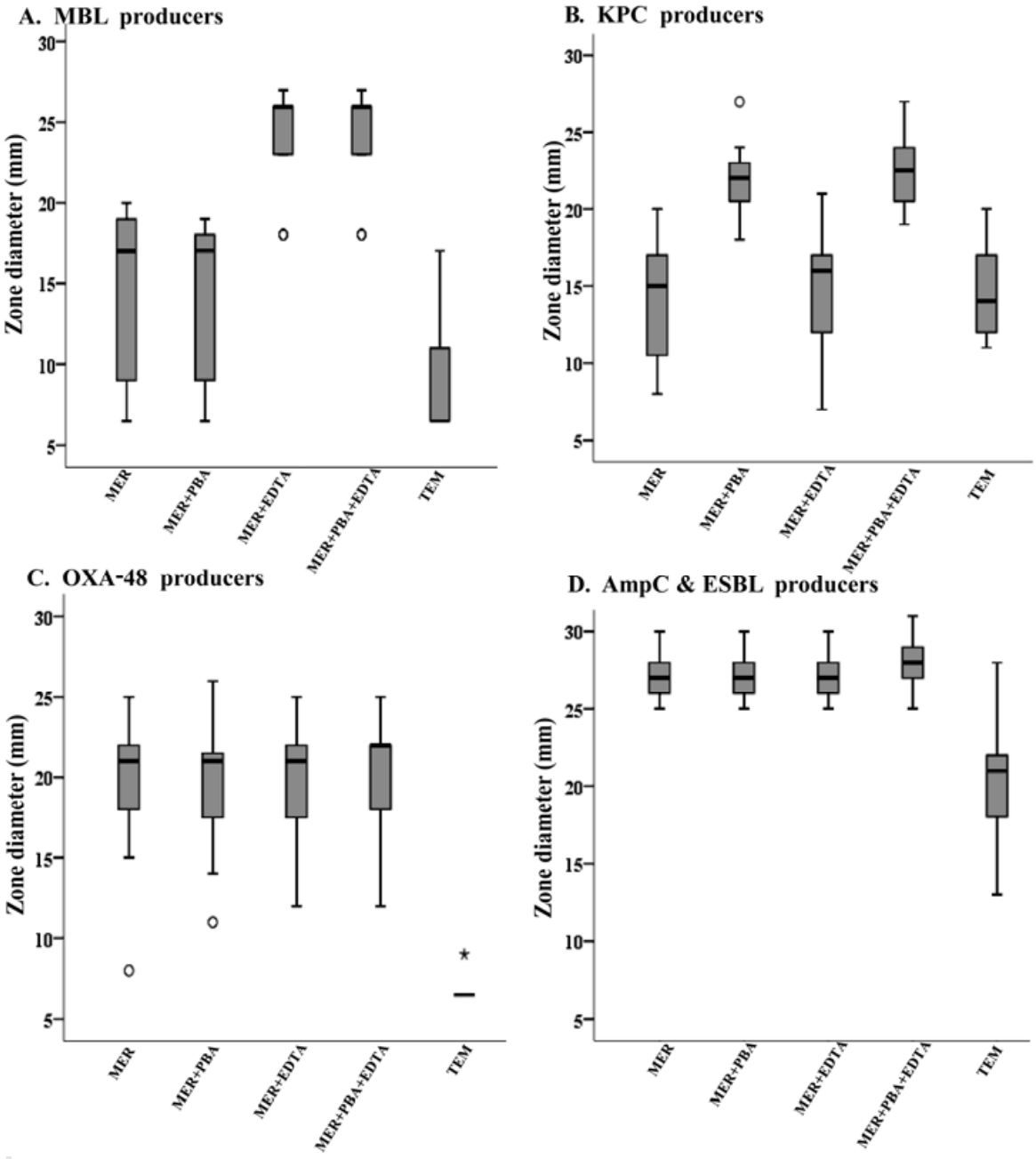
Figure legends

Fig. 1 Zone diameters of meropenem (MER) alone and with phenylboronic acic (PBA) and EDTA and temocillin alone.

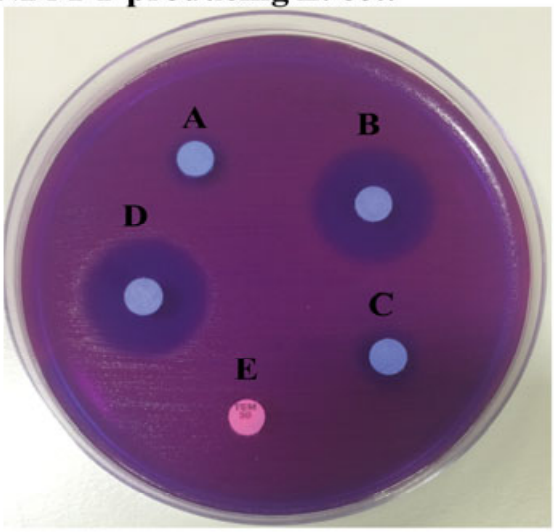
A = MBL producers (n=11); B = KPC producers (n=8); C=OXA-48 producers (n=11); D = non-carbapenemase producers (n=57). º = mild outlier; * extreme outlier.

Fig. 2 Phenotypic results from combined disc method along with RCA plate assay at 7 h.

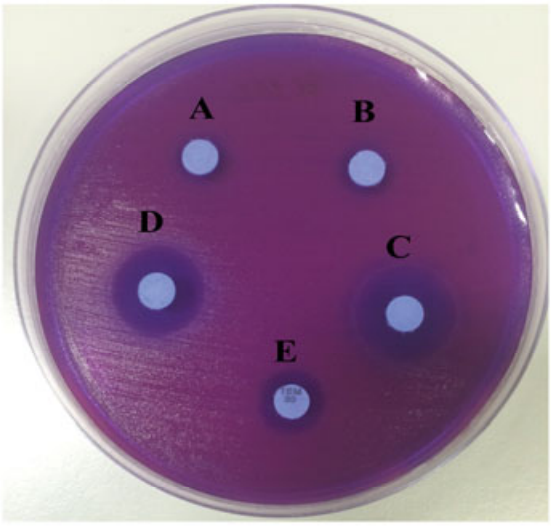
A = meropenem (10 µg); B = meropenem (10 µg) + EDTA (750 µg); C =meropenem (10 µg) + phenylboronic acid (400 µg); D = meropenem (10 µg) + EDTA (750 µg)+ phenylboronic acid (400 µg); E= temocillin (30 µg).



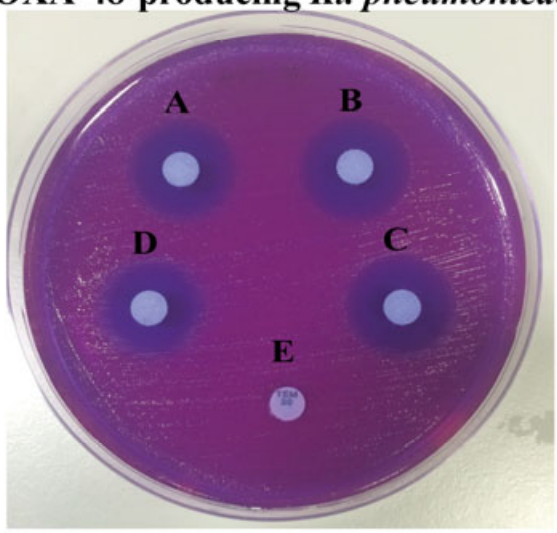
NDM-1-producing *E. coli*



KPC-4-producing *E. coli*



OXA-48-producing *Kl. pneumonieae*



CMY-2-producing *E. coli*