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Rapid Phenotypic Detection of AmpC β-Lactamases, Extended-Spectrum- β-Lactamases and Metallo-β-Lactamases in Enterobacteriaceae Using A Resazurin Microtitre Assay with Inhibitor-Based Methods

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Corresponding Author:	Katie Evans Liverpool John Moores University Liverpool, UNITED KINGDOM
First Author:	Yothin Teethaisong
Order of Authors:	Yothin Teethaisong
	Griangsak Eumkeb
	Sakesit Chumnarnsilpa
	Nongluk Autarkool
	Jon Hobson
	Ismini Nakouti
	Glyn Hobbs
	Katie Evans
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Abstract:	Dissemination of antibiotic resistance in Enterobacteriaceae mediated by AmpC, ESBL and MBL β-lactamases is clinically significant. A simple, rapid method for the detection of these resistance phenotypes would greatly improve chemotherapeutic recommendation. This technology would provide valuable input in our surveillance of resistance on a global stage, particularly if the methodology could be applicable to resource poor settings. A resazurin microtitre plate (RMP) assay incorporating cloxacillin (CX), clavulanic acid (CA), and EDTA for the rapid phenotypic identification of AmpC, ESBL, MBL and the co-existence of β-lactamases has been developed. A total of 44 molecularly characterised Enterobacteriaceae clinical isolates producing AmpCs, ESBLs, co-producers of ESBL and AmpC, MBLs, and co-producers of ESBL and MBL were phenotypically examined using the RMP assay. The ceftazidime (CAZ)-based and cefotaxime (CTX)-based RMP assay successfully detected all 15 AmpC, 12 ESBL, 9 MBL producers, 6 ESBL-AmpC co-producers, and 2 ESBL-MBL co-producers without false positive results. The CAZ-based assay was more reliable in detecting AmpC alone, while the CTX-based assay performed better in identifying co-producers of ESBL and AmpC. There was no difference in detection of ESBL and MBL producers. The findings of the present study suggest that use of the RMP assay with particular β-lactamase inhibitors explicitly detects three different β-lactamases, as well as co-existence of β-lactamases within 6 h. This assay is applicable to carry out in any laboratory, is cost-effective and easy to interpret. It could be implemented in screening patients, controlling infection and for surveillance purposes.

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Running title: AmpC, ESBL and MBL Detection Using Resazurin

Yothin Teethaisong[1,2],Griangsak Eumkeb[2], Sakesit Chumnarnsilpa[3] , Nongluk Autarkool[4], Jon Hobson[5], Ismini Nakouti[1], Glyn Hobbs[1], Katie Evans[1*]

[1] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, United Kingdom

[2]School of Pharmacology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand

[3]School of Biochemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand

[4]Faculty of Medical Science, Nakhon Ratchasima College, Nakhon Ratchasima, 30000, Thailand

[5]Mast Group Ltd, Bootle, Liverpool, L20 1EA, United Kingdom

* Address correspondence to Katie Evans

Email: K.Evans@ljmu.ac.uk, Tel.: +44-01512312334

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Abstract

Dissemination of antibiotic resistance in Enterobacteriaceae mediated by AmpC, ESBL and MBL β-lactamases is clinically significant. A simple, rapid method for the detection of these resistance phenotypes would greatly improve chemotherapeutic recommendation. This technology would provide valuable input in our surveillance of resistance on a global stage, particularly if the methodology could be applicable to resource poor settings. A resazurin microtitre plate (RMP) assay incorporating cloxacillin (CX), clavulanic acid (CA), and EDTA for the rapid phenotypic identification of AmpC, ESBL, MBL and the co-existence of β-lactamases has been developed. A total of 44 molecularly characterised Enterobacteriaceae clinical isolates producing AmpCs, ESBLs, co-producers of ESBL and AmpC, MBLs, and co-producers of ESBL and MBL were phenotypically examined using the RMP assay. The ceftazidime (CAZ)-based and cefotaxime (CTX)-based RMP assay successfully detected all 15 AmpC, 12 ESBL, 9 MBL producers, 6 ESBL-AmpC co-producers, and 2 ESBL-MBL co-producers without false positive results. The CAZ-based assay was more reliable in detecting AmpC alone, while the CTX-based assay performed better in identifying co-producers of ESBL and AmpC. There was no difference in detection of ESBL and MBL producers. The findings of the present study suggest that use of the RMP assay with particular β-lactamase inhibitors explicitly detects three different β-lactamases, as well as co-existence of β-lactamases within 6 h. This assay is applicable to carry out in any laboratory, is cost-effective and easy to interpret. It could be implemented in screening patients, controlling infection and for surveillance purposes.

Introduction

Infections caused by multidrug-resistant Enterobacteriaceae are among one of the most serious threats to human health (Tangden & Giske,2015). The production of β-lactamases mediated by both chromosomal and plasmid genes are crucial mechanisms of antibiotic resistance. AmpC β-lactamases were the first bacterial enzymes found to inactivate penicillin, first identified in *Escherichia coli* in 1940 prior to penicillins being medically introduced (Jacoby,2009). Plasmid-mediated AmpC β-lactamase (pAmpC)-producing Enterobacteriaceae can confer resistance to multiple antibiotics, such as penicillins, oxyimino-cephalosporins (e.g. cefotaxime, ceftazidime and ceftriaxone) and 7-α-methoxycephalosporins (e.g. cefoxitin and cefotetan) (Gupta *et al.*,2012). CMY-type β-lactamases, especially CMY-2 are the highest prevalence of pAmpC in *E. coli* that have been commonly identified in many regions of the world (Doi *et al.*,2010; Liebana *et al.*,2013). These enzymes are inhibited by boronic acid (BA), and its derivatives (Beesley *et al.*,1983) and cloxacillin (CX) (Jacoby,2009). AmpC β-lactamases are poorly inhibited by clavulanic acid (CA) (Bush *et al.*,1995).

Enterobacteriaceae can also produce powerful enzymes called extended-spectrum β-lactamases (ESBLs), which characteristically confer resistance to penicillins, cephalosporins, and monobactams, but remain susceptible to β-lactamase inhibitors (CA, tazobactam, sulbactam) cephamycins and carbapenems (Bradford,2001). The majority of ESBL-producing clinical isolates of Enterobacteriaceae harbour TEM-, SHV-, and CTX-M-type β-lactamases. These enzymes have gained medical significance in both hospital and community settings, across all ages and demographics (Lukac *et al.*,2015). Furthermore, carbapenem-inactivating β-lactamase, namely metallo-β-lactamase (MBL) has been identified with increasing prevalence over the past few years (Patrice *et al.*,2011). MBL can hydrolyse all classes of bicyclic β-lactams (penicillins, cephalosporins and carbapenems) and all

commercially available β-lactamase inhibitors (such as CA), but lack hydrolytic activity toward monocyclic β-lactams (monobactams) (Bebrone,2007). The activity of this enzyme is facilitated by zinc-ions to degrade β-lactams and is inhibited by metal-chelating ethylenediaminetetraacetic acid (EDTA) and dipicolinic acid (Walsh *et al.*,2005). Imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM) are common widespread MBLs. These metalloenzymes were frequently identified in *Pseudomonas aeruginosa* and *Acinetobacter* spp., but currently an increasing emergence of these have been documented in Enterobacteriaceae worldwide (Patel & Bonomo,2013).

The early detection of the presence of these resistant strains at the phenotypic level would aid infection-control practitioners to minimise the dissemination of these bacteria and also help to select appropriate antimicrobial treatment (Pitout & Laupland,2008). The development of a rapid method for the identification of these β-lactamase producing strains in clinical practice is a challenging issue. The resazurin (Alamar blue) reduction assay is a simple and inexpensive 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; Palomino *et al.*,2002). The present study uses the resazurin dye in conjunction with three distinct β-lactamase inhibitors, CX, CA, and EDTA, for the phenotypic detection of AmpC, ESBL, MBL and co-expression of β-lactamases.

Methods

Bacterial Isolates, Chemicals and Antibiotics

A total of 44 Enterobacteriaceae clinical isolates were used in this study. The bacterial strains included *E. coli* (n=13), *Klebsiella pneumonia*e (n=16) , *Enterobacter aerogenes* (n=2), *Enterobacter cloacae* (n=8), *Morganella morganii* (n=1), *Citrobacter freundii* (n=3), and *Klebsiella ozaenae* (n=1); 15 were AmpC producers, 12 were ESBL producers, 6 were ESBL and AmpC co-producers, 9 were MBL producers, and 2 were ESBL and MBL co-producers (Table 1). The control strains were *E. coli* ATCC 25922 (non-β-lactamase producer), *E. cloacae* ATCC BAA-1143 (bla_{ACT-32}), *E. coli* NCTC 13352 (bla_{TEM-10}) and *E. coli* NCTC 13353 ($bla_{CTX-M-15}$). All organisms had been identified by biochemical, phenotypic and molecular tests including combined disc, PCR and sequencing methods as previously described (Pérez-Pérez & Hanson,2002; Woodford,2010; Roschanski *et al.*,2014). All antibiotics and chemicals employed were purchased from Sigma Aldrich, UK. Mueller-Hinton Broth (MHB) powder was obtained from Scientific Laboratory Supplies, UK. Antibiotics and β-lactamase inhibitors were prepared in accordance with the recommendation of manufacturers.

Resazurin Microtitre Plate Assay (RMP)

The production of ESBL, AmpC, MBL and co-β-lactamases was phenotypically detected using the RMP assay based upon the broth microdilution method. An overnight culture of the appropriate test bacterium was pelleted for 10 min and washed twice with normal saline at 4,000 rpm for 5 min before adjustment to a 0.5 McFarland suspension according to the recommendations of the Clinical Laboratory Standards Institute (CLSI) in document M07-A9 (Clinical Laboratory Standards Institute,2012). An aliquot (20 µL) of a 5 x 10^6 CFU/mL of organism were added to the wells containing 180 µL of MHB, amended with resazurin at a concentration of 20 µg/mL, and serial twofold dilution of cefotaxime (CTX) or ceftazidime (CAZ) with a fixed concentration of β-lactamase inhibitors; CX at 200 µg/mL, EDTA at 0.5 mM, and CA at 4 µg/mL. Following incubation at 37 ºC, the lowest concentration showing

no colorimetric change from blue to pink within 6 h was considered as the MIC value. Interpretation criteria of the result obtained is explained in Table 2. The experiment was carried out in three independent replicates.

Results and Discussion

β-Lactam antibiotics are most frequently prescribed for the treatment of infections caused by Gram negative bacteria, in particular Enterobacteriaceae. Resistance to β-lactams is mediated by both plasmid- and chromosomally-encoded β-lactamases. The rapid global spread of these resistant bacteria is facilitated by inter- and intra-species gene transmission, poor sanitation and hygiene, increasing global trade, international travel and tourism, and antibiotic misuse (Rogers *et al.*,2011; Aung *et al.*,2012; Laxminarayan *et al.*,2013). The consequence is increased treatment failure and health risks. The development of rapid and accurate methods for the detection and characterisation of different types of β-lactamase remains an urgent necessity. The early detection is crucial in guiding appropriate antimicrobial therapy. The common conventional detection methods for the production of β-lactamase are still time-consuming (approximately 24 to 48 h) (Nordmann *et al.*,2012). Here, we developed the RMP assay with inhibitor-based methods for the early phenotypic detection of ESBL, AmpC, MBL, and co-production of β-lactamases.

The findings from the RMP assay were very simple and clear to interpret as illustrated in Figure 1. The results showed that the RMP CAZ-based assay was more reliable than the CTX-based assay for early AmpC detection. An eightfold or higher MIC ratio was observed when comparing the well without CX to other wells only for AmpC producers. Other MIC ratios revealed values lower than 8, which indicated negative results for ESBL and MBL producers. The CAZ-based assay successfully detected 15 of all 15 AmpC-producing

146 isolates, while 14 of 15 strains were detected by the CTX-based RMP assay. The AmpC
147 control strain clearly showed hyperproduction of AmpC β-lactamase indicated by a high MIC
148 value in the well without an AmpC inhibitor (Table 1). The production of AmpC β-lactamase
149 in Gram negative pathogens, especially Enterobacteriaceae is clinically important. Accurate
150 and rapid detection methods for these enzymes should be urgently developed. This would
151 ensure effective antimicrobial therapy and improve clinical management as a consequence of
152 clinical failure in the treatment of AmpC producers with broad-spectrum cephalosporins
153 (Polsfuss *et al.*,2011). Cloxacillin is well-described as an AmpC β-lactamase inhibitor, but it
154 cannot distinguish between plasmid-mediated and chromosome-borne AmpC (Jacoby,2009).
155 This study found that CAZ in conjunction with CX and EDTA was more sensitive for early
156 detection of AmpC-producing Enterobacteriaceae compared with the CTX-based assay. This
157 result is consistent with a previous report where CAZ showed the best performance compared
158 with CTX, cefmetazole, or moxalactam in combination with 3-aminophenylboronic acid
159 (APB) in detection of AmpC β-lactamases (Yagi *et al.*,2005). Another previous research
160 study reported no difference in sensitivity and specificity between CTX- and CAZ-based
161 detection in the presence of CA with and without BA in detection of pAmpC (Jeong *et*
162 *al.*,2009). In this study, some AmpC producers such as, *K. pneumoniae* 2001 (DHA-1), E.
163 coli 2002 (DHA-1), and *M. morganii* 2010 were partially inhibited by CX as indicated by the
164 high MIC values after exposure to CX. In those isolates we suggest that the resistance was
165 not only mediated by AmpC β-lactamase, but might also be conferred by alteration in the
166 permeability of the outer membrane.

167 The MIC ratio for wells without CA compared to wells with CA gave a value ≥8 in all tested
168 ESBL-producing Enterobacteriaceae employed in this study. The findings unambiguously
169 indicated that both CAZ-based and CTX-based assays gave reproducible results in detecting
170 all ESBL isolates. Even though CLSI has recommended a standardised confirmatory test for

ESBL-producing Enterobacteriaceae, previous reports found that using CTX and CAZ alone and in combination with CA demonstrated a high percentage of false-negative results in detection of ESBL producers due to co-production of β-lactamase enzymes (Jeong et al.,2009). In the current study inhibitor-based methods using three different β-lactamase inhibitors were applied to improve the sensitivity and specificity for phenotypic detection of ESBL in clinical isolates of Enterobacteriaceae. This modification allows the successful identification of all ESBLs in both CAZ-and CTX-based assays. Several techniques for detecting the production of ESBL have been proposed such as, double-disc synergy test, Etest and automated methods. The sensitivities and specificities for the double-disc test and Etest were 80% to 95%, whilst automated susceptibility methods were 80% to 99% (Drieux *et al.*,2008; Gazin *et al.*,2012). In addition, the ESBL NDP (Nordmann/Dortet/Poirel) test has been developed for rapid detection with an excellent sensitivity and specificity of 92.6% and 100%, respectively. However, this test was limited to detecting ESBL without presence of AmpC or other β-lactamases (Nordmann et al.,2012).

All MBL producers were detected by the RMP assay, including 9 MBL-producing isolates and 2 co-producers of ESBL and MBL. The CTX-based assay gave similar results with the CAZ-based assay for the rapid phenotypic detection of MBL producers. The MIC ratio of non-β-lactamase-producing *E. coli* ATCC 25922 for both CTX- and CAZ-based assays was 1, suggesting no ESBL, AmpC, and MBL production in this bacterium. A raised global dissemination of MBL-producing Enterobacteriaceae has drawn researchers to develop reliable techniques for detection of MBLs (Hattori *et al.*,2013). Yan and colleagues conducted double-disc, combined disc, and Etest techniques for the detection of Gram negative bacilli producing MBLs. They found that the 2-mercaptopropionic acid (2-MPA) double-disc test using CAZ and cefepime, with and without CA, exhibited highest sensitivity (100%) compared with the combined disc test (86.7%) and Etest (36.7%). Even the double-

196 disc test showed high sensitivity, but there were some disadvantages, including subjective
197 interpretation of results in some samples, and also volatile and odorous properties of 2-MPA
198 (Yan *et al.*,2004). More recently, double-disc synergy tests using sodium mercaptoacetic
199 acid (SMA) and EDTA as MBL inhibitors, and CAZ, imipenem and meropenem as indicator
200 antibiotics have emerged. The results showed that CAZ in combination with SMA can detect
201 only MBL-producing strains, whereas a combination of meropenem and SMA revealed the
202 best performance (sensitivity, 79.1%; specificity, 100%) in detection of co-production of β-
203 lactamases (Hattori et al.,2013). Yagi and colleagues also suggested the broth microdilution
204 method incorporating CAZ or CTX with SMA for successful detection of IMP-type MBL
205 (Yagi et al.,2005). Our findings revealed a good performance in the RMP assay for either
206 MBL alone or in combination with ESBL.

207 ESBL and AmpC co-producers were positively detected as indicated by a ≥8 MIC ratio for
208 wells containing either CA or CX compared to wells containing both CA and CX. The CTX-
209 based RMP assay was more reliable in phenotypic characterisation of the co-existence of
210 ESBL and AmpC β-lactamase compared with the CAZ-based RMP assay (Table 1). Co-
211 production of different types of β-lactamase in the same strain are one of the most common
212 causes of phenotypic detection failure. The most prevalent isolates co-producing pAmpCs
213 and ESBLs are *K. pneumoniae* and *E. coli* (Goossens & Grabein,2005). These co-β-
214 lactamases may result in false-negative tests for the detection of ESBLs or AmpCs
215 (Coudron,2005). A method that can distinguish between ESBLs and pAmpCs is important for
216 both treatment and epidemiology (Song *et al.*,2007). Furthermore, co-expression of MBL
217 together with ESBLs (commonly CTX-M-type) and AmpC β-lactamases (mostly CMY-
218 enzymes) appears to be difficult to detect. Consequently, these may be a major cause of false-
219 negative tests for detection of co-MBL, AmpC and ESBL in Gram negative pathogens
220 (Hattori et al.,2013; Tangden & Giske,2015).

Although simple phenotypic tests for ESBL, AmpC and MBL have been proposed by several researchers, and indeed conducted in clinical laboratory practice (Yagi et al.,2005; Jeong et al.,2009), there is no standard guideline for phenotypic detection of AmpC that has been recommended by the CLSI (Clinical Laboratory Standards Institute,2012). Broth microdilution methods have become more popular in clinical diagnostic laboratories since semi-automated antimicrobial susceptibility test systems have become commercially available. This test generally involves overnight incubation in the determination of antibiotic susceptibility profiling (Yagi et al.,2005). The present study using the RMP assay allows for the detection of ESBLs, AmpCs, MBLs and co-producers within 6 h. RMP assays could allow clinicians to detect these enzymes in rural clinical laboratories in resource poor settings.

Phenotypic tests for β-lactamase production are carried out in most clinical diagnostic laboratories because they are easy to implement and are cost effective. This detection is important in terms of epidemiological purposes and to restrict the dissemination of β-lactamase-mediated resistance mechanisms (Poulou *et al.*,2014). Phenotypic tests alone cannot distinguish between the specific *bla* genes of β-lactamases (TEM, SHV, CTX for ESBLs, CMY, MOX for AmpCs, and IMP, NDM, VIM for MBLs) (Pitout & Laupland,2008; Pitout *et al.*,2010), therefore, several reference laboratories use molecular methods for identification of those β-lactamase-encoded genes. Molecular approaches are restricted largely to reference laboratories for epidemiological studies because of the complexity and diversity of distinct point mutations of β-lactamases (Sundsfjord *et al.*,2004).

Although we used small samples of clinical isolates, a previous study found that use of CTX or CAZ combined with CA for detecting ESBL and CTX or CAZ plus BA for identifying pAmpC demonstrated 100% in both sensitivity and specificity (Jeong et al.,2009). In our study, the RMP assay detected all ESBL, AmpC, and ESBL-AmpC co-producers. Therefore,

we expect that our RMP assay could reach 100% in sensitivity and specificity similar to previous reports when a larger number of clinical isolates are tested. This assay can successfully distinguish co-expression of ESBL and AmpC or ESBL and MBL in the same strain. In future work, isolates with other resistance mechanisms, including alteration of permeability of bacterial membrane and modification of membrane proteins should be included in total isolates screened with RMP assay. The early detection of β-lactamase enzymes may reduce a delay in the initiation of appropriate antimicrobial therapy and could produce effective treatment. RMP assay is a useful method that can detect and characterise ESBL, AmpC, MBL and co-production of ESBL and AmpC or ESBL and MBL within 6 h. It could be implemented in microbiological diagnostic laboratories in resource poor areas to help clinicians screen patients, control infection, and provide epidemiological surveillance.

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Transparency declaration

The authors have no conflict of interest to declare.

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Figure 1 Schematic representation of results from resazurin microtitre plate assay at 6 h. CTX = cefotaxime; CAZ=ceftazidime; CA=clavulanic acid (4 µg/ml); CX = cloxacillin (200 µg/ml); EDTA = ethylenediaminetetraacetic acid (0.5 mM).

Figure

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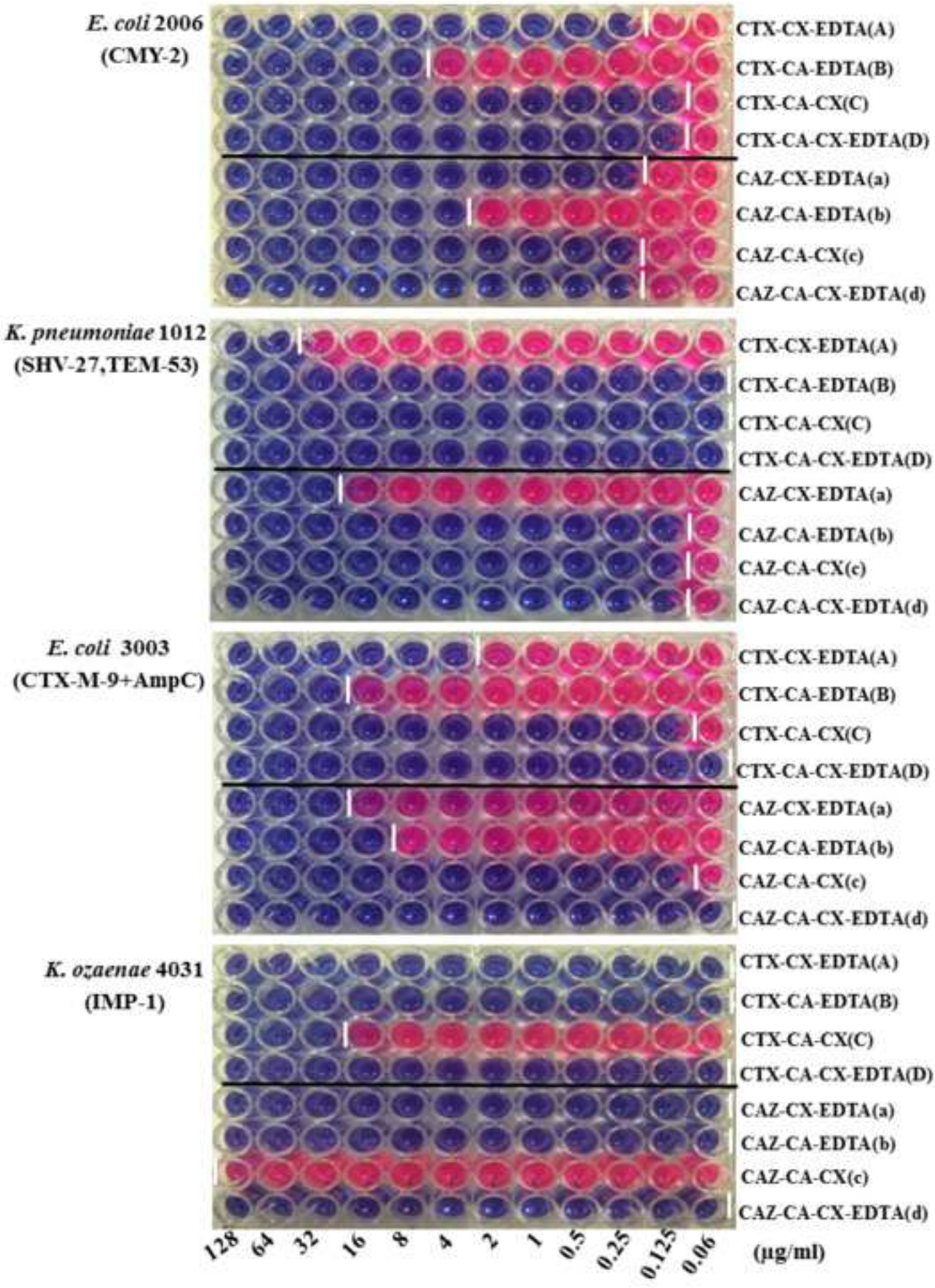

Table 1. Bacterial strains used in this investigation and results of phenotypic β-lactamase detection at 6 h using CTX -based[ABCD] and CAZ-based[abcd] assays.

Strains	Resistance Mechanism	MICs of CTX -based (µg/ml)				MICs of CAZ -based (µg/ml)				Phenotypic results
		A	B	C	D	a	b	c	d	
*_E. coli_ ATCC 25922	Negative control	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	None
K. pneumoniae 2001	DHA-1	4	≥256	8	4	8	≥256	16	8	AmpC
E. coli 2002	DHA-1	4	32	4	4	16	≥256	16	16	AmpC
E. coli 2003	AmpC	≤0.06	0.25	≤0.06	≤0.06	0.125	1	0.125	0.125	AmpC
E. coli 2004	CMY-2	≤0.06	32	≤0.06	≤0.06	≤0.06	64	≤0.06	≤0.06	AmpC
E. coli 2005	AmpC	≤0.06	2	≤0.06	≤0.06	0.125	4	0.125	0.125	AmpC
E.coli 2006	CMY-2	0.25	8	0.125	0.125	0.25	4	0.25	0.25	AmpC
E. aerogenes 2007	ACT-31	≤0.06	32	≤0.06	≤0.06	≤0.06	4	≤0.06	≤0.06	AmpC
E. cloacae 2008	FOX	≤0.06	16	≤0.06	≤0.06	0.125	32	0.125	0.125	AmpC
E. cloacae 2009	ACR-32	0.125	64	0.125	0.125	0.125	32	0.125	0.125	AmpC
M. morganii 2010	AmpC	2	64	2	2	8	≥256	8	8	AmpC
E. cloacae 2011	AmpC	≤0.06	64	≤0.06	≤0.06	≤0.06	32	≤0.06	≤0.06	AmpC
C. freundii 2026	CMY-112	≤0.06	32	≤0.06	≤0.06	≤0.06	32	≤0.06	≤0.06	AmpC
K. pneumoniae 2020	FOX	≤0.06	2	≤0.06	≤0.06	0.5	16	0.5	0.5	AmpC
E. coli 2021	FOX-3	≤0.06	0.5	≤0.06	≤0.06	≤0.06	8	0.125	0.125	AmpC
E. cloacae 2022	ACT-32	4	128	4	4	2	≥256	2	2	AmpC
*_E. cloacae_ BAA-1143	ACT-32	4	128	4	4	2	128	4	4	AmpC
E. coli 1004	TEM-214	64	0.125	0.125	0.125	16	0.25	0.25	0.125	ESBL
E. coli 1005	CTX-M-3	1	≤0.06	≤0.06	≤0.06	0.5	≤0.06	≤0.06	≤0.06	ESBL
E. coli 1006	CTX-M-3	64	0.125	≤0.06	≤0.06	1	0.125	0.125	0.125	ESBL
E. coli 1008	CTX-M-3	≥256	0.25	0.125	0.125	64	1	0.5	0.5	ESBL
E. coli 1009	TEM	32	0.125	≤0.06	≤0.06	4	0.125	0.125	0.125	ESBL
K. pneumoniae 1010	CTX-M-15,SHV-27	64	≤0.06	≤0.06	≤0.06	16	≤0.06	≤0.06	≤0.06	ESBL
K. pneumoniae 1012	SHV-27,TEM-53	64	≤0.06	≤0.06	≤0.06	32	0.125	0.125	0.125	ESBL
K. pneumoniae 1013	SHV-27	32	≤0.06	≤0.06	≤0.06	32	0.5	0.5	0.5	ESBL
K. pneumoniae 1015	SHV-27,TEM-71	16	≤0.06	≤0.06	≤0.06	8	0.125	0.125	0.125	ESBL
*_E. coli_ 13352	TEM-10	1	≤0.06	≤0.06	≤0.06	128	0.5	0.5	0.5	ESBL
*_E. coli_ 13353	CTX-M-15	128	≤0.06	≤0.06	≤0.06	32	≤0.06	≤0.06	≤0.06	ESBL
K. pneumoniae 1021	SHV-18	128	≤0.06	≤0.06	≤0.06	32	≤0.06	≤0.06	≤0.06	ESBL
K. pneumoniae 1022	SHV-27,TEM-115	32	≤0.06	≤0.06	≤0.06	16	≤0.06	≤0.06	≤0.06	ESBL
K. pneumoniae 1023	SHV-27,CTX-M-15	64	≤0.06	≤0.06	≤0.06	16	≤0.06	≤0.06	≤0.06	ESBL
E. cloacae 3007	CTX-M-9, MIR-1 ACT-1	16	64	≤0.06	≤0.06	1	32	≤0.06	≤0.06	AmpC+ESBL
E. cloacae 3009	TEM-214,SHV-12+ ACT	16	1	0.125	≤0.06	4	0.25	≤0.06	≤0.06	AmpC+ESBL
E. aerogenes 3022	SHV-12,CTX-M-9+ ACT-32	2	32	≤0.06	≤0.06	0.5	2	0.25	0.25	AmpC+ESBL
E. coli 3003	CTX-M-9+AmpC	4	32	0.125	≤0.06	32	16	0.125	≤0.06	AmpC+ESBL
C. freundii 3004	CTX-M-3+AmpC	2	32	≤0.06	≤0.06	0.25	4	≤0.06	≤0.06	AmpC+ESBL
C. freundii 3005	CTX-M-3+ ACT-1	4	32	≤0.06	≤0.06	32	4	0.25	0.25	AmpC+ESBL
K. ozaenae 4031	IMP-1	≤0.06	0.125	32	≤0.06	0.5	0.5	128	≤0.06	MBL
K. pneumoniae 4033	VIM-1+SHV-102	4	≤0.06	32	≤0.06	128	≤0.06	≥256	≤0.06	MBL+ESBL
K. pneumoniae 4034	VIM-1+SHV-12	8	≤0.06	8	≤0.06	128	≤0.06	128	≤0.06	MBL+ESBL
K. pneumoniae 4036	NDM-1	≤0.06	≤0.06	≥256	≤0.06	≤0.06	≤0.06	≥256	≤0.06	MBL
K. pneumoniae 4025	VIM-type	0.125	0.125	≥256	0.125	≤0.06	≤0.06	≥256	≤0.06	MBL
E. cloacae 4004	NDM-1	≤0.06	≤0.06	≥256	≤0.06	≤0.06	≤0.06	≥256	≤0.06	MBL
K. pneumoniae 4017	VIM-type	≤0.06	≤0.06	≥256	≤0.06	≤0.06	≤0.06	≥256	≤0.06	MBL
K. pneumoniae 4023	NDM-1	0.125	0.125	1	0.125	≤0.06	≤0.06	2	≤0.06	MBL
K. pneumoniae 4024	NDM-1	≤0.06	≤0.06	≥256	≤0.06	≤0.06	≤0.06	≥256	≤0.06	MBL
E. coli 4011	NDM-1	≤0.06	≤0.06	≥256	≤0.06	≤0.06	≤0.06	≥256	≤0.06	MBL
E. cloacae 4005	NDM	8	8	≥256	4	2	2	≥256	1	MBL

CTX= cefotaxime; CAZ= ceftazidime; CA= clavulanic acid (4 µg/ml); CX=cloxacillin (200 µg/ml); EDTA = ethylenediaminetetraacetic acid (0.5mM);A (a) = CTX (CAZ)+ CX+EDTA; B (b) = CTX(CAZ)+CA+EDTA;C (c) = CTX(CAZ)+CA+CX; D (d) = CTX (CAZ)+CA+CX+EDTA. * control strain

Table 2 Interpretation criteria of ceftazidime (CAZ)-based and cefotaxime (CTX)-based detection using resazurin microtitre plate assay.

MIC ratio ≥8	ESBL	AmpC	MBL
A/D or a/d	+	-	-
B/D or b/d	-	+	-
C/D or c/d	-	-	+
A/D or a/d and B/D or b/d	+	+	-
A/D or a/d and C/D or c/d	+	-	+
B/D or b/d and C/D or c/d	-	+	+
A/D or a/d and B/D or b/d and C/D or c/d	+	+	+

A (a) = CTX (CAZ)+ CX+EDTA
B (b) = CTX(CAZ)+CA+EDTA
C (c) = CTX(CAZ)+CA+CX
D (d) = CTX (CAZ)+CA+CX+EDTA