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Rapid detection of beta-lactamase production including carbapenemase by thin layer chromatography

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ABSTRACT

Objectives: To develop a rapid and simple method that can identify the presence of β-lactamases in clinical isolates and samples, and determine their activity on different types of β-lactam antibiotics, including carbapenems, within one hour.
Methods: In this study, we describe a thin layer chromatography-based method for rapid detection of β-lactamases including carbapenemases. The method relies on the examination of changes in the migration rate of β-lactams in chromatography, due to degradation by β-lactamase enzymes. A total of 44 isolates, 29 carbapenemase-producers and 15 non-carbapenemase-producers, were screened by this method.
Results: The method has proven to be able to distinguish β-lactamases as carbapenemase or non-carbapenemase producing strains with high sensitivity in one hour.
Conclusions: The method developed, provides information about the production of β-lactamases by bacteria and β-lactam drugs inactivated by these enzymes, including carbapenems. This new method may play an important role in guiding antimicrobial treatment, especially in critically ill patients infected bacteria producing β-lactamases.

1. Introduction

β-Lactam antibiotics are important drugs for the treatment of serious infections. However, in recent years, a rapid increase in β-lactamase producing strains has limited their use (Kong et al., 2010; Deshpande et al., 2004; Jeon et al., 2015). As new groups of β-lactam antibiotics are introduced for medical use, β-lactamases have evolved to degrade these new β-lactams. Among these β-lactams, carbapenems have remained active against β-lactamases for a much longer time after introduction for medical use, in contrast to other β-lactams (Papp-Wallace et al., 2011). However, with the emergence of new enzymes called carbapenemases, carbapenem resistance has also become widespread (Gupta et al., 2011; Queenan and Bush, 2007; Nordmann et al., 2011). Rapid detection of carbapenemase production is crucial for rapid determination of carbapenem resistance. There are several phenotypic (Seah et al., 2011; Miriagou et al., 2010; Nordmann et al., 2012a; Tsakris et al., 2009; Samra et al., 2008; Galani et al., 2008), molecular and biochemical techniques (Kaase et al., 2012; Endimiani et al., 2010; Cuzon et al., 2012; Spanu et al., 2012) for detection of carbapenemase production. Phenotypic techniques, such as the modified Hodge test require overnight incubation (Pasteran et al., 2010). DNA amplification based techniques can rapidly detect β-lactamase genes, however, they cannot detect all of these genes with a single test due to high variability in these genes (Diene and Rolain, 2014). Immunochromatographic tests like Resist-3-O.K.N. (Coris BioConcept, Gembloux, Belgium) can detect OXA-48, KPC and NDM type enzymes with 100% sensitivity, although it cannot detect IMP and VIM type enzymes (Wareham and Momin, 2017). Current commercially available rapid biochemical tests, such as Carba NP and Blue-Carba, are easy, rapid and reliable (Pires et al., 2013; AbdelGhani et al., 2015; Nordmann et al., 2012b; Bakour et al., 2015). These acidometric tests are designed to detect carboxylic acid formation after the hydrolysis of the β-lactam ring of a carbapenem, by observing a color change of a pH indicator.

From a clinical point of view, when a bacterial strain producing β-lactamase is detected it is more important to know the β-lactams that are inactivated by this enzyme rather than the type of the enzyme, so that the treatment regimen can be chosen accordingly. The aim of this study was to develop a simple universal test that can determine which

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Table 1
Results of Lactamaster test on carbapenemase and noncarbapenemase-producing strains.

Carbapenemase-producing strains (no. of isolates)	Type of enzyme	Antimicrobial Hydrolysis				
		CFZ	CXM	CRO	CTX	ETP
K. pneumoniae (1)	KPC	+	+	+	+	+
K. pneumoniae (2)	KPC	+	+	+	+	+
K. pneumoniae (3)	KPC	+	+	+	+	+
K. pneumoniae (4)	KPC	+	+	+	+	+
K. pneumoniae (5)	NDM-1	+	+	+	+	+
K. pneumoniae (6)	NDM-1	+	+	+	+	+
K. pneumoniae (7)	NDM	+	+	+	+	+
E. coli (1)	NDM-1	+	+	+	+	+
K. pneumoniae (8)	NDM + OXA-48	+	+	+	+	+
K. pneumoniae (9)	VIM	+	+	+	+	+
K. pneumoniae (10)	VIM	+	+	+	+	+
P. aeruginosa (1)	VIM-4	+	+	+	+	+
E. coli (2)	IMP	+	+	+	+	+
K. pneumoniae (11)	IMP	+	+	+	+	+
E. cloacae (1)	IMP	+	+	+	+	+
K. pneumoniae (12)	IMP	+	+	+	+	+
K. pneumoniae (13)	IMP	+	+	+	+	+
K. pneumoniae (14)	OXA-48	+	+	+	+	+ [a]
K. pneumoniae (15)	OXA-48	+	+	+	+	+ [a]
K. pneumoniae (16)	OXA-48	+	+	+	+	+ [a]
K. pneumoniae (17)	OXA-48 + SHV1	+	+	+	+	+ [a]
Acinetobacter baumannii (1)	OXA-51 + GES	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (2)	OXA-51 + GES + OXA-58	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (3)	OXA-51 + GES + OXA-23	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (4)	OXA-51 + OXA-23	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (5)	OXA-51 + OXA-24	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (6)	OXA-51 + OXA-24 + OXA-23	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (7)	OXA-51 + OXA-58	+	± [c]	+	± [c]	+ [a]
Acinetobacter baumannii (8)	OXA-51 + OXA-58 + OXA-23	+	± [c]	+	± [c]	+ [a]
Non-carbapenemase producing strains (no. of isolates)						
E.coli (3)	CTX-M-8	+ [b]	+ [b]	+ [b]	+ [b]	- [b]
E.coli (4)	CTX-M-15	+ [b]	+ [b]	+ [b]	+ [b]	- [b]
E.coli (5)	CTX-M-15	+ [b]	+ [b]	+ [b]	+ [b]	- [b]
E.coli (6)	CTXM-1	+ [b]	+ [b]	+ [b]	+ [b]	- [b]
E.coli (7)	CTX-M-3	+ [b]	+ [b]	+ [b]	+ [b]	- [b]
E. coli (8)	TEM-3	+	+ [d]	+ [d]	+ [d]	–
K. pneumoniae (18)	TEM-4	+	- [d]	- [d]	- [d]	–
K. pneumoniae (19)	SHV-31	+	–	–	–	–
K. pneumoniae (20)	SHV-31	+	–	–	–	–
K. pneumoniae (21)	CTX-M-3 + SHV-11	+	+	+	+	–
K. pneumoniae (22)	CTX-M3 + TEM-1 + SHV-1 + SHV-5	+	–	–	–	–
K. pneumoniae (23)	CTX-M-15,SHV-1, OXA-48	+	–	–	–	–
K. pneumoniae (24)	CTX-M-15,TEM-1,SHV-1	+	–	–	–	–
E. coli (9)	CMY-2	+	+	+	–	–
K. pneumoniae (25)	ACT Group	+	+	+	+	–

[a] OXA-48 produces two degradation products of ETP as a characteristic property.
[b] CTX-M produces tailing spots on the TLC plates.
[c] Microorganisms which produce a combination of β-lactamases instead of one type of enzyme, test results demonstrated a different pattern for CXM and CTX.
[d] TEM-3 producers yielded positive results, whereas TEM-4 yielded negative results for CXM, CRO, CTX.

types of β-lactams are inactivated by β-lactamases detected in bacterial strains. For this purpose, we designed a novel test to demonstrate the presence of β-lactamases including carbapenemases, based on detection of hydrolysis products of β-lactams, using thin layer chromatography, which we called "Lactamaster" (patent pending).

2. Materials and methods

2.1. Bacterial isolates

Lactamaster was used to test a collection of Gram-negative bacilli ($n = 44$) which produced specific β-lactamases: 29 carbapenemase producing isolates harbouring IMP, VIM, NDM, KPC or OXA-48 genes, and 13 isolates producing non-carbapenemase, TEM, SHV, CTX-M, and 2 isolates producing pAmpCs, CMY and ACT (Table 1). The collection was previously characterized by determination of MIC for carbapenems using the microdilution assay according to EUCAST criteria and identification of β-lactamase genes by multiplex PCR (Cakar et al., 2016).

2.2. Antibiotics

In this study, we used four cephalosporin derivatives from different generations; cefazolin (CFZ), cefuroxime (CXM), ceftriaxone (CRO), cefotaxime (CTX) and one carbapenem derivative, ertapenem (ETP). Antibiotics were obtained from Sigma-Aldrich (St. Louis, United States). Stock solutions of antibiotics (3 mg/mL) were prepared in deionized water, distributed into portions in a 96-well plate and dried in a level 2, biosafety cabinet. The reason we chose these antibiotics was that they all migrated at a different rate in a non-overlapping manner on a chromatography plate and they were affected differently by different

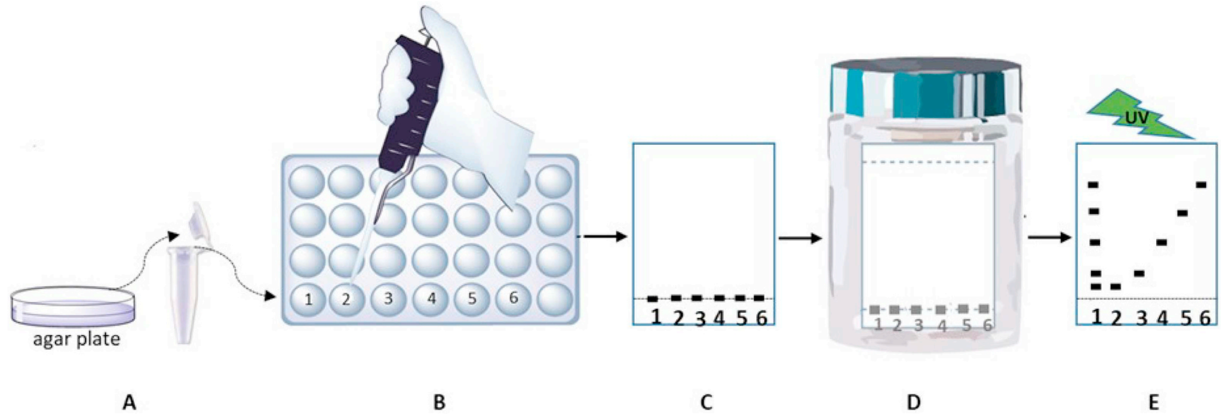

Fig. 1. Representative application of Lactamaster test. **A.** The tested bacteria from a solid culture are diluted in a microcentrifuge tube, in deionized water **B.** β-Lactam antibiotics in a 96-well tray. The first well includes a mixture of the antibiotics, and the following wells are for cefazolin (CFZ), cefuroxime (CXM), ceftriaxone (CRO), cefotaxime (CTX) and one carbapenem derivative, ertapenem (ETP), respectively. **C.** TLC plate with a starting line at the bottom, which has six loading points. **D.** The container including a shallow layer of solvent. **E.** TLC plate is visualized under UV light, showing the migration distance of antibiotics from the starting line.

types of β-lactamases. Additional β-lactams, like ceftazidime (CAZ), may be added to the test for further characterization of β-lactamase activity.

2.3. Incubation

For the detection of β-lactamases by Lactamaster, a loopful of the tested bacteria taken from solid culture medium by a sterile 10-ul disposable plastic loop, was diluted in a microcentrifuge tube with 500 μL of deionized water. From this suspension 100 μL was mixed with five different β-lactam antibiotics in a 96-well tray, in which these antibiotics were previously dried. The final concentration of all antibiotics was 0.6 mg/mL. These bacteria-antibiotic mixtures were incubated at 37 °C for 30 min. Following incubation, 3 μL of the samples were applied on thin layer chromatography plates (Fig. 1A). Incubations were performed in duplicate, and for each, three replication of thin layer chromatographic analysis were applied.

2.4. Thin layer Chromatography (TLC) method

The TLC plates (20 × 20 cm) (Merck, Darmstadt, Germany) consisted of aluminum foil coated with a thin layer of silica gel as adsorbent material. The plates were cut to 3 × 5 cm pieces. A starting line, with a distance of 5 mm to the bottom edge, was drawn using a pencil and six loading points were marked on this line. To the first loading point a mixture of five antibiotics without any bacteria, which will be used as marker, was loaded (Fig. 1). Each of the following five points was loaded with one of the bacteria-antibiotic mixtures. After the samples were loaded, the plate was immersed in a container, including a shallow layer of solvent (ethyl acetate-water-acetic acid mixture, 6:2:2). (Ethyl acetate and acetic acid were purchased from Sigma-Aldrich). It was important that the solvent level was shallow, below the starting line, where the samples were applied. The plate was kept standing for 10 min in the container. While, the solvent slowly moved up the plate by capillary action, it drived the samples through the plate. The intact and degraded components of the antibiotic molecules, which have affinities for the silica, migrated at different rates. When the solvent completed its movement, the plate was taken out from the container and dried at room temperature. It was possible to dry the plates faster using a hair dryer without affecting the results. The antibiotics on the plate were visualized under ultraviolet light at 254 nm. Alternatively, the plate was colored by dipping it into potassium permanganate solution (1.5 g $KMnO_4$, 10 g K_2CO_3, 1.25 mL 10% NaOH in 200 mL water) for one minute and dried, to visualize antibiotics.

3. Results

We first determined the behaviour of intact antibiotics (CFZ, CXM, CRO, CTX, ETP) on the TLC plate before mixing them with any bacteria. Each antibiotic produced a single clean spot with specific retardation factors (R_F) (Fig. 2A).

Incubation of antibiotics with a strain lacking β-lactamase as a control, *Escherichia coli* ATCC 25922, produced the same R_F pattern as the marker in chromatograms (Fig. 2B). Although these antibiotics have an unstable nature, contact with silica of TLC plate, solvents used in the experiment and mixing with bacterial cultures of β-lactamase negative strains, did not cause any degradation. An example showing the visualization of plates using a staining solution (potassium permanganate) instead of UV light, is included in Fig. 2F.

Incubation of antibiotics with a carbapenemase-producing strain, caused the disappearance of the initial spots of CFZ, CXM, CTX, corresponding to the marker, whereas spots of CRO, ETP migrated to a different position, with a different R_F value (Fig. 2C). Organisms producing OXA-48 enzymes produced two spots after degradation of ETP, which can be used as a characteristic property on TLC to identify the presence of this enzyme (Fig. 2D). These changes in the chromatograms indicated that all five of the antibiotics, including ETP, were degraded by any type of carbapenemase enzyme (Table 1). However, in the presence of a β-lactamase other than carbapenemase, ETP was not degraded (Fig. 2E). Degradation of five antibiotics was analyzed with different bacterial strains producing different types of carbapenemase enzymes (Table 1). All 29 carbapenemase-producers (NDM, KPC, IMP, VIM and/or OXA) gave positive results and all 13 non-carbapenemase or pAmpC producing strains gave negative results for ETP degradation. Carbapenemase producers degraded all the other β-lactam drugs tested which belonged to 1st, 2nd and 3rd generation cephalosporins, except some strains that produced OXA-48 which did not degrade ceftazidime. Among strains shown to contain single or even multiple non-carbapenemase β-lactamase genes of different types, some degraded only cefazolin.

β-Lactam antibiotics other than CFZ, CXM, CRO, CTX, ETP could be also used in our test, according to need. For example, some carbapenemases do not degrade ceftazidime (CAZ) (Wiskirchen et al., 2014). In order to demonstrate this, we incubated CAZ with six different bacterial strains producing TEM-3, KPC, VIM, OXA-48, NDM-1, or IMP. The results of the test showed that, TEM-3 and OXA-48 did not degrade CAZ (Fig. 3). Use of ceftazidime for our test may produce valuable data for treating patients infected with carbapenem-resistant strains, which may be susceptible to ceftazidime.

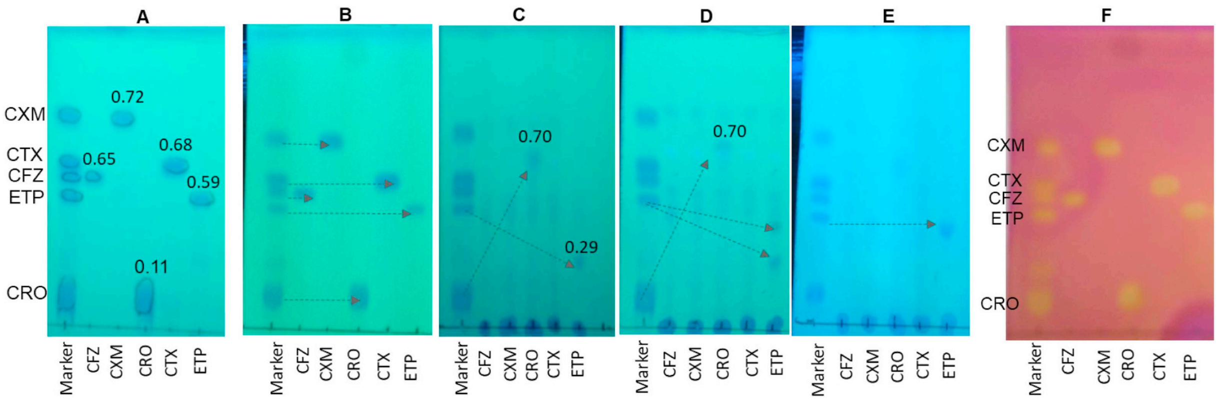

Fig. 2. Representative results of the Lactamaster test. A: TLC plate of intact antibiotics; R_F (CFZ) = 0.65; R_F (CXM) = 0.72; R_F (CRO) = 0.11; R_F (CTX) = 0.68; R_F (ETP) = 0.59 **B:** TLC plate after antibiotics were incubated with *E. coli* ATCC 25922. **C:** TLC plate after antibiotics were incubated with a KPC-producing *K. pneumoniae*. Degradation products of CFZ, CXM, CTX were invisible under UV light. R_F Values of degraded CRO and ETP are 0.70 and 0.23, respectively. **D:** TLC plate after antibiotics were incubated with a OXA-48-producing *K. pneumoniae*. **E:** TLC plate after antibiotics were incubated with a SHV-producing *K. pneumoniae*. **F:** A TLC plate colored using potassium permanganate solution, after antibiotics were incubated with *E. coli* ATCC 25922. R_F: Retardation factor. CFZ: cefazolin, CXM: cefuroxime, CRO: ceftriaxone, CTX: cefotaxime, ETP: ertapenem.

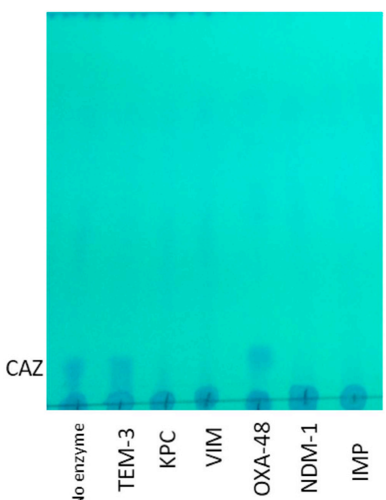

Fig. 3. Representative TLC results of the ceftazidime (CAZ) incubated with different types of carbapenemases. CAZ was incubated with different enzymes from left to right; no enzyme (control), TEM-3 (*E.coli*), KPC (*K. pneumoniae*), VIM (*K. pneumoniae*), OXA-48 (*K. pneumoniae*), NDM-1 (*K. pneumoniae*), and IMP (*K. pneumoniae*).

4. Discussion

Rapid detection of β-lactamases in pathogens causing serious infections is very important in order to choose the right empirical treatment regimen, until antibiotic susceptibility test results become available. Determination of the type of β-lactamase is also crucial since it helps to estimate which drugs will be inactivated by the enzyme. Rapid detection of carbapenemase producing isolates is a clinical necessity since the gene responsible for enzyme production is almost always located on transferable plasmids (Diene and Rolain, 2014), which can easily spread in hospitals and cause outbreaks, if required infection control measures are not strictly followed.

There are several genotypic and phenotypic methods for rapid identification and typing of β-lactamases (Seah et al., 2011; Miriagou et al., 2010; Nordmann et al., 2012a; Tsakris et al., 2009; Samra et al., 2008; Galani et al., 2008). These tests detect effectively carbapenemase activity but do not identify the activity of the enzyme against other β-lactam drugs like ceftazidime, which is not always inactivated by carbapenemases like OXA-48 (Pasteran et al., 2010). This may be critical information for treating patients with ceftazidime, who are infected by these kinds of carbapenem-resistant strains. Real-time PCR based methods like X-pert Carba R (Cepheid, Sunnyvale, CA, USA) achieved detection of five different types of carbapenemases, NDM, IMP, VIM, KPC and OXA-48 with high sensitivity (Tato et al., 2016). Identifying a gene of β-lactamase does not always guarantee that the gene is expressed to produce the enzyme (Jeon et al., 2015). Additionally high variability in β-lactamase enzymes changes the activity differently against different beta-lactam drugs. Some of the strains we have analyzed in this study did not inactivate β-lactams other than cefazolin, although the presence of 3 or 4 types of β-lactamases was identified previously by PCR.

In this study we have developed a test, which detects the presence of β-lactamases and their activity against different β-lactam compounds including carbapenems, using thin-layer chromatography. Like in some strains used in this study, the presence of β-lactamase genes does not always guarantee that they will show activities expected against some β-lactam drugs (Pires et al., 2013; AbdelGhani et al., 2015; Nordmann et al., 2012b; Bakour et al., 2015). From a clinical point of view, it is more important to show the activity of the enzyme than identifying its type. In many cases bacteria contain more than one type of β-lactamase, which makes it harder to estimate the activity of the combination of these enzymes. The new test that we have developed has the advantage of rapid identification of activity of the β-lactamases against different β-lactams, which is crucial in guiding the therapy of infections caused by pathogens producing these enzymes.

A number of publications regarding the identification of β-lactams by TLC have previously appeared in literature just for the purpose of molecular characterization, however, none of these aimed to identify the presence of carbapenemase enzymes (Hancu et al., 2013; Mendez et al., 2011). The method we have developed in this study identifies carbapenemase-producing strains within 1 h with 100% specificity. In addition, the method allows screening of which β-lactams are inactivated by a tested clinical isolate.

In conclusion, the assay we have developed in this study allows screening clinical isolates for their enzymatic activity including carbapenemases. Further validation of this assay, especially to determine its sensitivity, in different clinical isolates with varying levels of enzymatic activity, is needed, to demonstrate its clinical utility. The efficiency of direct detection of β-lactamases, in samples like urine or culture media of positive blood culture bottles, by this new method, is under investigation.

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We have no conflict of interest to declare.

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