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Outline of a bacterial filter-based assay to detect beta-lactamases

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ABSTRACT

We describe a new phenotypic test to detect beta-lactamases. This assay is based on diffusion of beta-lactam/beta-lactamase through a bacterial filter. Beta-lactam hydrolysis on (the other side of) the filter leads to a change in antibiotic susceptibility, which can be measured by disc diffusion tests. We illustrate its ease of use to detect beta-lactamases of different classes.

Introduction

The beta-lactam antibiotics have been amongst the most successful drugs for the treatment of bacterial infections for the past 60 years (Coleman, 2011). Resistance to antimicrobials is emerging worldwide at an alarming rate amongst various bacterial species (Livermore, 2012), causing both community-acquired and nosocomial infections (Spellberg et al., 2011).

More than half of all currently used antibiotics belong to the beta-lactam group, but their clinical effectiveness is severely compromised by the emergence of beta-lactam-resistant bacteria (Allen et al., 2010). Therefore correct detection of extended-spectrum beta-lactamases (ESBL) and carbapenemases is crucial for antibiotic choice and infection control.

Many techniques can be used for detecting these beta-lactamases: from phenotypic methods to microarray (Wintermans et al., 2013) or matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (Knox et al., 2014) or the now more commonly used molecular-based techniques, e.g. PCR and gene sequencing (Reuland et al., 2013 and Poirel, et al., 2011). All genotypic tests are resistance gene-specific, therefore choosing the incorrect test leads to false negative results. Phenotypical tests, in contrast, are often easy to perform, cheap, and less specific. Because they are less specific, they can be used to detect a wide range of resistance mechanisms like porin loss, presence of efflux pumps, and beta-lactamase production. The downside is that co-occurrence of multiple resistance mechanisms in the same strain can hamper detection of beta-lactamase production (Nordmann et al., 2012).

The Modified Hodge Test (MHT), which can detect a wide range of beta-lactamases, is commonly used but can be difficult to perform and to interpret by inexperienced technicians (Carvalhaes et al., 2010).

The Carba NP test (Tijet et al., 2013) is a hydrolysis assay, but it is a dedicated carbapenemase test.

A commonly used phenotypical test is the double disc combination test (Rosco Diagnostica A/S, Taastrup, Denmark). This test performs well on a wide range of beta-lactamases (Wintermans et al., 2013 and Van Dijk et al., 2014) but detection is depended on the blocking of specific resistance mechanisms, this detour can hamper the results and will fail in absence of correct/specific inhibitors.

We describe a new phenotypical principle to detect beta-lactamases; tests based on this principle can be performed in a classical clinical laboratory with commonly available resources. In this study we tested beta-lactamase-producing strains from different groups to demonstrate this.

Methods

For the phenotypical assays we used test strains with well-known genetic characteristics, of which two strains are American Type Culture Collection Quality control strains (http://www.lgcstandards-atcc.org) and five strains were tested by PCR and gene sequencing in earlier studies (Reuland et al., 2015 and Van Dijk et al., 2014) (Table. 1). These strains harbour resistance genes (CTX-M, VIM, OXA-48, NDM) to hydrolyse various antibiotics from differ classes. All the test strains were collected from storage (−70 °C) and cultured on standard blood agar (Becton Dickinson, Sparks, MD, USA).

The principle of MHT (Lee et al., 2001) is to visualize beta-lactamases by testing susceptibility of a susceptible *E. coli* strain (ATCC 25922) in the presence of a beta-lactamase-producing bacterium. In a positive MHT the beta-lactamase hydrolyses the antibiotic that has been applied

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Table 1
Strains used in this study.

Test	Assay	Species	Code	Figure	phenotype	Genotype
1	MHT	*Klebsiella pneumoniae*	JS132	1-1	Class D carbapenemase	OXA-48
2	Filter	*Klebsiella pneumoniae*	JS132	4	Class D carbapenemase	OXA-48
3	MHT	*Klebsiella pneumoniae*	RC-21	1-2, 5-1	Class B metallo-beta-lactamase	VIM-1
4	Filter	*Klebsiella pneumoniae*	RC-21	5	Class B metallo-beta-lactamase	VIM-1
5	Filter	*Klebsiella pneumoniae ATCC*	BA1706	3	wildtype	wildtype
6	Filter	*Klebsiella pneumoniae*	TY8858	6	Extended-spectrum beta-lactamase	CTX-M-15
7	Filter	*Klebsiella pneumoniae*	JS022	7	Class B metallo-beta-lactamase	NDM-1
8	All	*Escherichia coli ATCC*	25922	All	Wildtype	wildtype

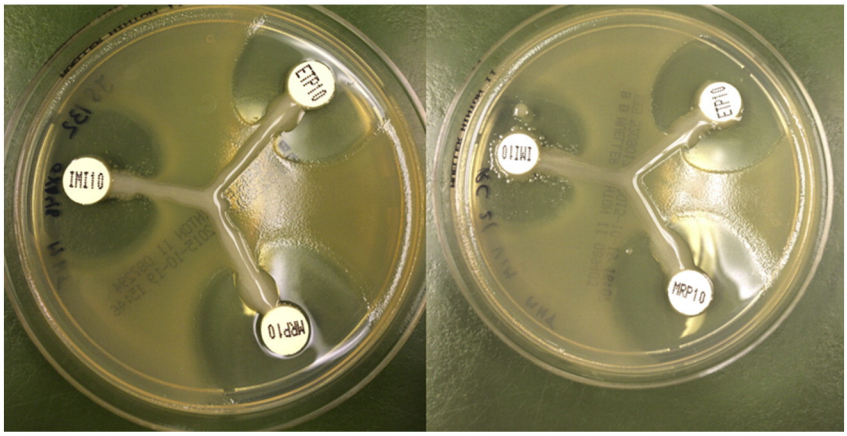

Fig. 1. On the left side an easy to interpret MHT with a well recognizable cloverleaf effect (*K. pneumoniae* OXA-48). The right side shows a less obvious test result, in which interpretation can lead to debate (*K. pneumoniae* VIM-1).

on the Mueller Hinton (MH) agar, this leads to a locally lower antibiotic concentration and therefore to distortion of the inhibition zone of the susceptible *E. coli*. We used the MHT to detect OXA-48 and VIM-1 from *Klebsiella pneumonia*.

The filter test differs from the MHT: the two test strains are separated by a bacterial filter so that diffusion of beta-lactamase from the producing strain to the susceptible strain has its effect over a much larger area, thereby leading to a larger effect in deformation of inhibition zones and therefore to easier and more sensitive interpretation. Also antibiotics diffuse through the filter and are hydrolysed by the resistant strain on the opposite side of the filter leading to lower antibiotic concentrations on both sides of the filter.

To achieve this we used a Mueller Hinton agar (Becton Dickinson, Sparks, MD, USA), standard antibiotic discs (Rosco Diagnostica), standard 0.45-μm filter (Sartorius Stedim Systems, Germany), a reference *Escherichia coli* strain (ATCC 25922), and a random strain to test for beta-lactamase activity.

By drawing a line on the back of the plate we separated the MH-agar in two sides. On one side a pure culture of the resistant test strain (i.e. *Klebsiella pneumoniae* OXA-48, as used in the MHT) was applied. Then the filter was placed on top of the plate so that only one half of the filter had beta-lactamase producing strain underneath. A suspension of *E. coli* (ATCC 25922) was applied over the whole filter and an antibiotic disc of choice was placed in the middle. We used the discs: meropenem, ertapenem and imipenem, comparable to the discs used in the MHT.

We also performed the test with a carbapenemase negative *Klebsiella pneumoniae* (ATCC BA1706) to illustrate a negative assay.

The plates were incubated overnight at 37 °C before interpretation. During incubation the antibiotic spreads over the plate by diffusion but is hydrolysed by the beta-lactamase strain; in addition, free beta-lactamase will diffuse through the filter leading to hydrolysis on the other side of the filter. These two processes both lead to lower local antibiotic concentrations and result in a difference of inhibition-zone of the *E. coli* on top of the filter. The bacterial growth on the filter was tested by matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to rule out bacterial growth trough the filter.

To illustrate its broad applicability we performed the filter test on a collection of strains that produced beta-lactamase from different groups: extended spectrum beta-lactamases and different carbapenemases (Table 1). All tests were performed in duplicate.

Adding discs, which inhibit specific beta-lactamases, can help in further specification of the results.

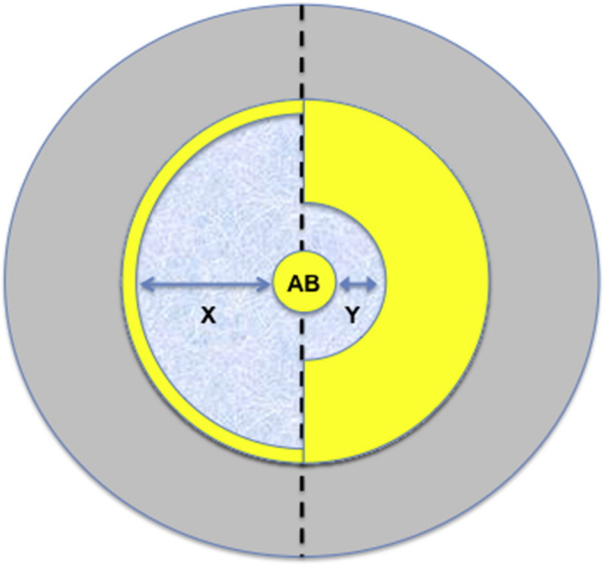

Fig. 2. Schematic representation of a positive filter test: 'X' marks the inhibition zone of the beta-lactamase free side and 'Y' the side where beta-lactamase is exposed to the antibiotic resulting in a smaller zone.