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Short Communication

Rapid detection of cefotaxime-resistant *Escherichia coli* by LC–MS

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ABSTRACT

Antibiotic resistance is an unsolved healthcare problem with increasing impact on patient management in the last years. In particular, multidrug resistance among Gram-negative bacterial strains has become the most pressing challenge. In order to deliver the most efficacious antimicrobial therapy with minimum delay, rapid diagnostic tests are required in order to detect multidrug resistant pathogens early during infection. In line with these efforts, we have developed a mass spectrometry-based assay for the rapid determination of ampicillin and cefotaxime resistance. The assay quantifies beta-lactamase activities towards ampicillin and cefotaxime within a turnaround time of 150 min, which is substantially faster than classical susceptibility testing.

1. Introduction

In cases of sepsis, early knowledge-based de-escalation of the antimicrobial broad spectrum therapy reduces the spread of antimicrobial resistances (Fraser et al., 2006; Goldmann et al., 1996; McGowan, 1994) and is shown to reduce therapeutic costs (Beekmann et al., 2003; Coleman et al., 1991; Tumbarello et al., 2010). However, commercially available culture-based antibiotic susceptibility testing (AST) e.g. Vitek 2 requires 9.82 ± 2.32 h to generate a complete microbial report including antibiotic susceptibility results for Gram-negative rods (Gherardi et al., 2012). In cases of severe sepsis, a delay of antimicrobial therapy for only few hours is associated with high case fatality rates (Ferrer et al., 2014). Rapid susceptibility testing is thus urgently needed.

To accelerate microbial diagnostics, we have previously demonstrated a liquid chromatography-mass spectrometry (LC–MS) based assay to detect antibiotic susceptibilities and resistances (MAAST – mass spectrometry-based antibiotic susceptibility testing). The test detected resistance of *Escherichia coli* towards ampicillin within 90 min after microbial growth has been detected in blood cultures (Grundt et al., 2012).

MS-based assays for susceptibility testing rely upon monitoring of the microbial biotransformation of antibiotics (Hooff et al., 2012; Sparbier et al., 2012; Wimmer et al., 2012). This metabolism of antibiotics results in a mass shift of the antibiotics which can rapidly be detected by mass spectrometry.

In contrast to the qualitative MALDI-TOF MS approaches (Sparbier et al., 2012; Wimmer et al., 2012) MAAST is a combination of high performance liquid chromatography (HPLC) and mass spectrometry, which is capable to separate and quantify multiple compounds simultaneously. This setting allows the reproducible identification and quantification of compounds, such as antibiotics and their inactive metabolites. The compound concentration directly correlates with the signal intensity of the compound-specific mass at the respective separation time. Accordingly, LC–MS/MS can exactly quantify the native beta-lactam antibiotics as well as the hydrolysis products for susceptibility testing of beta-lactam antibiotics.

2. Material and methods

2.1. HPLC and MS

The HPLC separation was performed using an Agilent series 1100 LC system (Agilent Technologies) with a Zorbax Eclipse

Abbreviations: AST, antibiotic susceptibility testing; AMP, ampicillin; AUC, area under the curve; CAZ, ceftazidime; CLSI, Clinical and Laboratory Standards Institute; CTX, cefotaxime; *E. coli*, *Escherichia coli*; HPLC, high performance liquid chromatography; LC, liquid chromatography; MAAST, mass spectrometry-based antibiotics susceptibility testing; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MIC, minimal inhibitory concentration; MS, mass spectrometry; SPZ, sulfaphenazole.

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XDB-C18 column (Agilent Technologies) and a constant gradient from 0 to 83% of buffer B within 14 min. The composition of buffer A was 2 mM ammoniumformiate with 0.1% formic acid and buffer B was acetonitrile with 0.1% formic acid. The flow rate was set to 400 μl/min. During the complete separation time we continuously collected MS-data by an amaZon Speed mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization (ESI) source. To serve as nebulizer and drying gas we used nitrogen. Helium was used for collision-induced dissociation, which is required for the unique identification of masses by dissociation into specific fragments.

Firstly, we optimized LC–MS conditions for sensitive and specific quantitation of antibiotics using selected reaction monitoring (SRM). The mass transitions of cefotaxime (CTX) and the internal standard sulfaphenazole (SPZ) were found to be m/z 456.0 → 396.0 for CTX and m/z 315.0 → 221.8 for SPZ (Fig. 1A and B). MS-spectra were recorded in a mass window ranging from m/z 50 to 800 using the UltraScan mode. The linearity and precision for CTX and SPZ quantitation using selected reaction monitoring (SRM) was analyzed in a concentration range of 0.01–10 μg/ml. The coefficient of determination (R^2) for the quantification of CTX was greater than 0.99 (Fig. 1C) with a coefficient of variation smaller than 15% for any tested concentration.

2.2. Incubation

Bacteria were obtained by sub-cultivation for 12 h at 37 °C prior to MAAST. For the susceptibility testing of CTX with subcultured *E. coli*, we incubated the bacteria in Optimem 1 Medium (Life Technologies) at a density of 4.0 McFarland standard with a mixture of 1 μg/ml CTX. The incubation time for the detection of CTX hydrolysis was set to 2 h and to 5 h additionally at a temperature of 37 °C. After clearing by centrifugation for 3 min at 6000 × *g* the internal standard SPZ was added at a concentration of 0.1 μg/ml. After another centrifugation for 10 min at 17,000 × *g*, CTX and the internal standard SPZ were simultaneously quantified in the supernatant by LC–MS within a 14-min HPLC-gradient and the ratio of CTX and SPZ was calculated using the signal intensities of the respective fragments.

The assay was evaluated with 105 clinical *E. coli* isolates that had been characterized previously by a Vitek 2 instrument delivering a MIC. The Vitek 2 instrument characterized 39 isolates to be CTX-susceptible (MIC ≤1 μg/ml, CLSI) and 66 isolates to be CTX-resistant (MIC ≥4 μg/ml, CLSI). Additionally the resistance was confirmed by the molecular detection of the CTX-M gene.

3. Results

Using the MAAST protocol with 120 min incubation time and a cut off ratio of 32.5 for CTX/SPZ, we found a sensitivity (resistant-tested among resistant) of 92.4% and a specificity (susceptible-tested among susceptible) of 97.4% (results compared to Vitek 2) (Fig. 2A) The false negatives are explained by the fact that some *E. coli* isolates appear to hydrolyze CTX very slowly. Concordant with this, we found increased incubation periods of 5 h resulted in a sensitivity of 92.4% and a specificity of 100% (Fig. 2B). For the prolonged incubation time we used a cut off ratio of 21.0 for CTX/SPZ due to spontaneous hydrolysis during the prolonged incubation time resulting in lower overall signals for CTX.

The resistant isolates that were classified false negative, because of high remaining amounts of CTX also had low MICs of 8 μg/ml detected by Vitek 2 (Fig. 2C and D). Furthermore, these isolates were susceptible for CAZ, while all other CTX-resistant isolates were CAZ-resistant too (data not shown). This shows that these isolates have beta-lactamases of low-activity in metabolizing cephalosporins. These beta-lactamases belonged to the molecular type CTX-M. Even if the isolates with a MIC of 8 μg/ml still cannot be detected after 5 h, their CTX/SPZ ratios differ more strongly from those of susceptible strains than they do after 2 h (Fig. 2C and D).

Among the 66 *E. coli* isolates, which were classified to be CTX-resistant by Vitek 2, we found 61 isolates to be classified as multidrug resistant, as they showed resistances against three or more types of antibiotics (ureidopenicillins, cephalosporins and fluoroquinolones). Among these multidrug resistant Gram-negative bacteria, we detected CTX-resistance in 58 cases within a 2 h incubation time (sensitivity of 95.0%, specificity of 97.4%). The increased incubation time of 5 h delivered a sensitivity of 95.0% and a specificity of 100% among multidrug resistant isolates. This can be explained by higher enzyme activities among multidrug resistant isolates resulting in a lower number of false negatives in this selected group.

4. Discussion

The main focus of this study is the reliable identification of all CTX-resistant *E. coli* isolates by MAAST. Although this cannot be achieved with the low hydrolyzing strains, the gain in time for the detection of multidrug-resistant *E. coli* isolates is still substantial when compared to standard culture-based procedures with incubation times ranging up to approximately 12 h (Gherardi et al., 2012). The MAAST protocol takes a total time of 2.5 h for the short incubation time. The preceding time for the sub-cultivation ist the same as in conventional methods. An alternative approach to reduce the time of analysis might be the serial quantification of CTX and SPZ from one sample (e.g. every 30 min), which allows for both, the early detection in fast metabolizing bacteria as well as secure detection in slow metabolizing bacteria. Other studies have shown that Vitek-2 also might deliver information about antibiotic resistance in a short time from 3.3 to 17.5 h by testing directly from positive blood cultures (Bruins et al., 2004; Ling et al., 2003). To keep up with these assays it has to be shown that MAAST CTX testing can also be performed with bacteria directly obtained from blood cultures like it has been shown for AMP (Grundt et al., 2012). This requires a highly sensitive method for CTX and SPZ quantification, as is the case with the LC–MS used in this study. Qualitative MS-based approaches for the detection of CTX resistance will not be suitable for this kind of AST testing and further on, might be unable to detect the slow metabolizing strains even after a 5 h incubation time. For further clinical evaluation we suggest a multisampling approach including the AMP testing as described before (Grundt et al., 2012).

MALDI-TOF based AST is not able to reproducibly quantify compounds with high precision required for this application (Sparbier et al., 2012). The moderate reproducibility of quantitative analyses is a major limitation of MALDI-TOF MS and has been demonstrate by many groups (e.g. Szájli et al., 2008). However, the use of internal standards is alleviating many problems and a rapid antibiotic resistance testing for meropenem using MALDI-TOF MS has been reported recently (Lange et al., 2014). Nevertheless, LC–MS is the method of choice for therapeutic drug monitoring and therefore was chosen for quantitation of CTX in this study. The high precision of the LC–MS/MS-based quantification of antibiotics and metabolites with a coefficient of variation of <15% is the key advantage of this method compared to the qualitative MS-based AST-assays. This can be ensured by the introduction of several quality control samples in each run. This precision is essential for a rapid and safe detection of antibiotic resistances especially in slow metabolizing bacterial isolates. This might not only reduce costs of therapy, but