Rapid molecular detection of extended-spectrum \(\beta\)-lactamase gene variants with a novel ligation-mediated real-time PCR

Roel Nijhuis,¹ Anton van Zwet,¹ James Cohen Stuart,² Thijs Weijers³ and Paul Savelkoul⁴

INTRODUCTION

Extended-spectrum \(\beta\)-lactamases (ESBLs) are emerging worldwide, making rapid and adequate ESBL detection crucial for infection control measures as well as for the choice of correct antimicrobial therapy. The aim of this study was to compare the performance of a novel rapid ligation-mediated real-time PCR (LM-PCR) with a combination disc test (CDT). In total, 172 prospective putative ESBL-positive Enterobacteriaceae isolates from clinical specimens based on VITEK2 results were included in this study and tested with the phenotypic CDT and the LM-PCR. Positive ESBL results were obtained in 100 and 95 isolates using CDT and LM-PCR, respectively. The sensitivity, specificity, negative predictive value and positive predictive value of the LM-PCR were 99.0, 92.2, 98.6 and 94.0 %, respectively, compared with the CDT. The LM-PCR technique provides an important reduction in turnaround time (~4.5 h versus overnight incubation using CDT) for ESBL confirmation. As a consequence, all ESBL results are available within the same day, making this assay an important tool for rapid and accurate ESBL detection.
screened positive for ESBL production according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2009) by VITEK2 using AST cards 140 (for urine samples) and 141 (for all other materials) (bioMérieux) at the Department of Medical Microbiology and Medical Immunology (Rijnstate Velp, The Netherlands) and were included in this study. Isolates originated from clinical specimens, of which only the first of each different ESBL-positive Enterobacteriaceae species (by VITEK2) was included.

DNA extraction. For all putative ESBL-positive isolates determined by VITEK2, DNA extraction (NucliSens EasyMAG, protocol specific A with 110 μl DNA eluate; bioMérieux) was performed using 200 μl of a 0.6–0.8 McFarland suspension of pure culture, including a blank control sample. Before DNA extraction, phocine herpesvirus 1 (PhHV-1) DNA was added to each suspension at a final concentration equivalent to a standard threshold cycle (Ct) value of −32 ± 2 (± SD) in the real-time PCR (van Doornum et al., 2003).

LM-PCR. The LM-PCR is based on single-nucleotide polymorphism detection, using the previously described Check-ESBL assay (Check-Points), and is able to detect the most prevalent ESBL types (CTX-M1 group, CTX-M2 group, CTX-M9 group, SHV-238S, TEM-104K and TEM-164S) (Cohen Stuart et al., 2010; Endimiani et al., 2010; Nijhuis et al., 2011). Fig. 1 shows a schematic presentation of the principle of LM-PCR. In short, a ligation step (non-cyclic: 3 min at 95 °C, 2 h at 65 °C, 2 min at 95 °C) was performed using 5 μl solution A, 2.5 μl LpE mix (including probes with an ESBL gene-specific part, a molecular beacon-binding site and a ZIP code for putative subsequent microarray analysis (Cohen Stuart et al., 2010)) and 10 μl DNA. After the ligation step, real-time PCR was performed in a 30 μl volume containing 2× TaqMan Universal Mastermix (Applied Biosystems), RTe solution (Check-Points) containing generic primers and a fluorescently carboxyfluorescein-labelled molecular beacon, PhHV-1 primers and a fluorescently Cy5-labelled PhHV-1 TaqMan probe (Biolegio) together with 5 μl ligation product using the TaqMan 7500 system (Applied Biosystems).

To exclude false-positive results due to background signals (Ct values >37), a cut-off value was introduced using the internal control PhHV-1, for which the titration values are well known. The ESBL LM-PCR was considered positive when the ESBL signal was stronger than the PhHV-1 signal (ESBL Ct value< PhHV-1 Ct value) using a threshold of 0.05 and an automatic baseline (automatically correcting the background fluorescence detected), according to the manufacturer’s guidelines.

Phenotypic characterization. Phenotypic ESBL detection was performed according to standard protocols with a combination disc test (CDT), as described previously (Carter et al., 2000; Garrec et al., 2011). In short, discs containing a cephalosporin (cefotaxim, ceftriaxone) and a combination of this cephalosporin with clavulanic acid were placed on Mueller–Hinton agar inoculated with 0.5 McFarland suspension of the isolate. Isolates were considered positive if the inhibition zone, measured after overnight incubation at 37 °C, around the combination disc (cephalosporin + clavulanic acid) was at least 5 mm larger than the corresponding cephalosporin disc.

Discrepancy testing. Discrepant results between the CDT and LM-PCR were analysed for the presence of TEM, SHV and CTX-M ESBLs using a conventional PCR and subsequent sequencing, as well as by a Check-MDR CT103 assay (Check-Points).

The conventional PCR and sequencing for molecular characterization of ESBLs were performed as described by Cohen Stuart et al. (2010) and were able to detect the ESBL genes TEM, SHV and CTX-M.

The Check-MDR CT103 assay has been well evaluated by Cuzon et al. (2012) and is able to detect most prevalent ESBLs (blaTEM, blaSHV and blaCTX-M1), plasmid-mediated AmpC β-lactamases (blaCMY-2,blaCMY-4,blaOXA-48,blaACC-1,blaACT/TMPR and blaiti-MOX) and carbapenemases (blaKPC, blavIM-MB, blavaMD and blaNDM).

RESULTS

A total of 172 putative ESBL-producing Enterobacteriaceae were identified with VITEK2 and further analysed by CDT and LM-PCR. Isolates were obtained from 163 patients, from a total of 168 clinical specimens (117 from urine, 17 from rectal swabs/faeces, seven from sputum, six from positive blood cultures and 21 from miscellaneous specimens), and represented a total of ten bacterial species, most of which were Escherichia coli (101/172; 58.7%) (Table 1).

Positive ESBL results were obtained in 100 (58.1%) and 95 (55.2%) isolates using CDT and LM-PCR, respectively. No further differentiation between ESBL groups was determined because the currently tested molecular beacon was designed to detect only the generic ESBL groups (Fig. 1). In all LM-PCR tests, no inhibition was detected based on the correct PhHV-1 DNA real-time PCR signal (mean Ct value of PhHV-1 in the negative control ± 2 SD).

Comparing the results of CDT and LM-PCR, seven ESBL discrepancies were found, of which one isolate was detected that was CDT-negative/LM-PCR-positive. This LM-PCR-positive result was confirmed by both conventional PCR with subsequent sequencing and the Check-MDR CT103 assay, detecting CTX-M group 1 ESBL and non-ESBL TEM. Of the remaining six discrepancies (CDT-positive/LM-PCR-negative), all LM-PCR-negative results could be confirmed using conventional PCR and sequencing and the Check-MDR CT103 assay. Using the Check-MDR CT103

---

Fig. 1. Principle of LM-PCR. (a) Annealing (5′-cPrimer-1 MB ZIP cSeq-1 cPrimer-2), connecting the two probe arms. (c, d) Real-time PCR is performed using generic primers (binding to sites cPrimer-1 and cPrimer-2) and a molecular beacon (MB) (d), using a generic molecular beacon-binding site (c-MB). The ZIP sequence, which is specific for each probe, can be used for microarray analysis.
assay, in three of these isolates (two Enterobacter cloacae and one Escherichia coli), an AmpC gene (ACT/MIR in both Enterobacter cloacae isolates and CMY-2 in the Escherichia coli isolate) was detected, and in two Escherichia coli strains, a non-ESBL TEM was found; no resistance genes were detected in the remaining Escherichia coli (Table 1).

The sensitivity, specificity, and positive and negative predictive values of the LM-PCR compared with the CDT were 99.0, 92.2, 94.0 and 98.6 %, respectively (Table 2).

**DISCUSSION**

This study describes the first evaluation of a new LM-PCR method (Check-Points) that is able to detect all important amino acid substitutions (TEM and SHV) and genes (CTX-M) responsible for the most prevalent ESBLs. The present study compared this LM-PCR with a conventional phenotypic CDT.

Of the 172 putative ESBL-producing Enterobacteriaceae, seven discrepancies were found when we compared the CDT and LM-PCR. Discrepancy testing of CDT-positive/LM-PCR-negative isolates confirmed all six LM-PCR-negative results. In three of the six isolates, an AmpC gene was detected (two Enterobacter cloacae and one Escherichia coli combined with a non-ESBL TEM), a β-lactamase hyper-producer with similar patterns of resistance to extended-spectrum cephalosporins such as ESBL that is able to generate false-positive results in phenotypic ESBL tests by producing an increase in the inhibition zone with the combination cephalosporin–clavulanic disc (Kohner et al., 2009; Roberts et al., 2009).

Furthermore, two Escherichia coli isolates harboured a non-ESBL TEM (TEM-1), of which high expression has previously shown false-positive ESBL phenotypes in phenotypic ESBL confirmation methods (Beceiro et al., 2011). Wu et al. (2001) found that decreased outer-membrane permeability, due to loss of outer-membrane protein K35 (OmpK35), and the hydrolytic effect of TEM-1 can increase the MIC of cefotaxim slightly. The addition of clavulanic acid inhibits TEM-1 β-lactamase production and reduces the MIC, causing augmentation of the inhibition zone with the combination cephalosporin–clavulanate disc (Kohner et al., 2009; Roberts et al., 2009).

The remaining CDT-positive/LM-PCR-negative ESBL result (Escherichia coli) could not be confirmed by either conventional PCR or Check-MDR CT103 (ESBL-, AmpC- and carbapenemase-negative). The LM-PCR is an easy-to-perform assay that should be considered positive when the Gt value of the ESBL is less than the Gt value of the PhHV-1 control and negative when the Gt value of the ESBL is greater than the Gt value of the PhHV-1 control because of signals in the real-time PCR (around cycle 37 and higher) caused by non-specific binding during the ligation step. Testing pure cultures of clinical isolates and using the well-known titration values of PhHV-1 as the cut-off value

<table>
<thead>
<tr>
<th>Isolate</th>
<th>CDT positive</th>
<th>LM-PCR positive</th>
<th>Discrepancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrobacter freundii</td>
<td>7</td>
<td>74</td>
<td>18</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>6</td>
<td>71</td>
<td>11</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>18</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>101</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>11</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>70</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>172</td>
<td>95</td>
<td>95</td>
</tr>
</tbody>
</table>

*Discrepancy testing was performed using PCR and sequencing and the Check-MDR CT103 assay.
Table 2. Results of the CDT and LM-PCR

<table>
<thead>
<tr>
<th>Result</th>
<th>LM-PCR positive</th>
<th>LM-PCR negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDT positive</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>CDT negative</td>
<td>1</td>
<td>71</td>
</tr>
</tbody>
</table>

with the LM-PCR gives a clear signal to noise discrimination ratio between ESBL-specific and non-specific signals.

Wrong (subjective) interpretation of the CDT on borderline results was probably the reason for the CDT-negative *Escherichia coli* that tested positive using LM-PCR, conventional PCR and Check-MDR CT103. Queenan et al. (2004) found false-negative results in phenotypic ESBL screenings using a lower range of standard inoculum, including the *Klebsiella pneumoniae* ATCC 700603 SHV ESBL control strain, which could also explain the phenotypic-negative, gene-positive *Escherichia coli*.

The LM-PCR and the methods for discrepancy testing (PCR followed by sequencing and the Check-MDR CT103 assay) detect only the prevalent ESBL genes TEM, SHV and CTX-M. Therefore, it could not be excluded that the few LM-PCR-negative/CDT-positive ESBLs were the result of rarer ESBL genes (e.g. PER, VEB or GES). Our evaluation concentrated on detection of the most prevalent ESBL genes and it was beyond the scope of this study to determine other putative genes. In addition, this assay had a high sensitivity and negative predictive value (99.0 and 98.6%, respectively) compared with the CDT, which demonstrates a reliable performance for routine diagnostics, comparable to the currently used microarrays. Moreover, based on our findings, we believe that CDT probably leads to false-positive results, confirming the results of Garrec et al. (2011) in which the CDT gained a specificity ranging from 80 to 91% (depending on the antibiotics used) compared with PCR. Therefore, the specificity of the LM-PCR will probably be even greater than 92.2%.

The LM-PCR analysis time of about 4.5 h (45 min DNA extraction, 2 h ligation, 1 h 40 min real-time PCR) is considerably shorter than that required for the CDT, as the latter requires an overnight incubation.

The ability to detect transmissible antibiotic resistance, responsible for increased morbidity and mortality and prolonged hospital stays, one day earlier could positively influence the effectiveness of infection control (Kluytmans-Vandenbergh et al., 2005; Quirante et al., 2011; Song et al., 2009). Rapid laboratory detection is necessary to take appropriate infection control measures, according to the Dutch guidelines for preventing nosocomial transmission of highly resistant micro-organisms. Moreover, rapid and correct detection of ESBLs is important to guide correct antimicrobial therapy (Kluytmans-Vandenbergh et al., 2005; Willemsen et al., 2011).

Although the use of LM-PCR is more expensive than CDT (catalogue price €19.80 versus ~€1.20 per sample, respectively), it should be emphasized that the substantial number of false-positive ESBL results using the CDT will lead to unnecessary isolation of patients. It has been calculated that isolation of a patient means an additional €26.34 each day on a regular ward in a non-outbreak situation (Wassenberg et al., 2010).

Looking at other recently developed molecular methods to detect the most prevalent ESBLs, LM-PCR has several advantages. For example, the turnaround time of the LM-PCR (~4.5 h) is shorter than that of the microarray Check-ESBL assay (6.5–8 h, depending on the number of samples) (Cohen Stuart et al., 2010; Nijhuis et al., 2011).

Both Voets et al. (2011) and Dallenne et al. (2010) evaluated a conventional set of (multiplex) PCRs to detect the most important β-lactamases without the use of an internal control. These methods, as well as the microarray system, have a large disadvantage for routine application because of a risk of contamination due to a post-amplification step (using amplification products for gel electrophoresis or microarray hybridization) that is needed for detection. Under routine laboratory conditions, these tests are applied on cultured strains; therefore, detection with a closed amplification system such as real-time PCR has major advantages.

In conclusion, the LM-PCR appears to be an accurate method to rapidly detect the most prevalent ESBLs, TEM, SHV and CTX-M. The assay can easily be introduced into any laboratory with real-time PCR equipment, allowing rapid detection of ESBLs. In fact, with only a few laboratory workflow adaptations, a new important reduction in turnaround time for molecular ESBL screening can be established. As a consequence, all ESBL results would be available within 1 day, making this assay an important tool for rapid and accurate ESBL detection.

ACKNOWLEDGEMENTS

We thank the laboratory diagnostic team for collecting the clinical isolates, and Marc Zegers and Brenda Slotboom for their excellent technical assistance. We would also like to thank Check-Points for technical support and for providing the material necessary for this study. This research was performed partly within the framework of the Center for Translational Molecular Medicine (CTMM) (http://www.ctmm.nl/pro1/general/home.asp), project MARS (grant 041-201). This work was supported by Check-Points BV, Wageningen, The Netherlands.

REFERENCES


ESBL ligation-mediated real-time PCR assay


