Rapid detection of CTX-M-producing Enterobacteriaceae in urine samples

Cynthia Oxacelay†, Ayla Ergani†, Thierry Naas* and Patrice Nordmann

Service de Bactériologie-Virologie, INSERM U914: ‘Emerging Resistance to Antibiotics’, Hôpital de Bicêtre, Assistance Publique-Hôpitaux de Paris, Faculté de Médecine, Université Paris-Sud, 94275 Le Kremlin-Bicêtre, France

Received 24 June 2009; returned 27 July 2009; revised 18 August 2009; accepted 19 August 2009

Objectives: CTX-M extended-spectrum β-lactamases (ESBLs) are emerging worldwide. Fast and reliable detection techniques may become mandatory for implementing proper treatment and infection control measures. Here, a blaCTX-M-specific LightCycler real-time PCR (LC-PCR) assay based on hybridization probes was developed.

Methods: Urine samples positive for Gram-negative bacilli as revealed by Gram staining were collected over a 3 month period at Bicêtre hospital, France. Aliquots of these urine samples were frozen for subsequent molecular analysis, and the bacteria were cultured and identified by standard bacteriological techniques (biochemical tests, disc diffusion antibiogram and synergy testing). LC-PCR and standard PCR followed by sequencing was performed on all ESBL-positive and on 70 randomly chosen ESBL-negative urine samples.

Results: Over the study period, 810 urine samples were collected from 655 patients. Thirty-six ESBL-producing Enterobacteriaceae, mostly Escherichia coli (77%), were identified from 29 patients, of which half were outpatients. Twenty-five urine samples (19 patients) were found to be positive for blaCTX-M genes using the LC-PCR assay. The blaCTX-M genes belonged to the blaCTX-M-1, blaCTX-M-9 and blaCTX-M-2 groups (68%, 24% and 8%, respectively). Standard PCR and sequencing of the entire blaCTX-M genes confirmed the LC-PCR results: 17 CTX-M-15, 6 CTX-M-9 and 2 CTX-M-2. Among the remaining ESBLs, eight were of the TEM type and three of the SHV type.

Conclusions: The LC-PCR assay represents a powerful tool for rapid identification of CTX-M producers in urine samples.

Keywords: ESBLs, detection, real-time PCR, hybridization probes

Introduction

Plasmid-encoded extended-spectrum β-lactamases (ESBLs) of the CTX-M type are reported increasingly worldwide in Gram-negative bacilli (GNB).1,2 CTX-M now account for most of the ESBLs found in Enterobacteriaceae. More than 80 variants have been described and are divided into five groups based on amino acid sequence identity (groups CTX-M-1, -2, -8, -9 and -25), with different groups prevalent in different countries. ESBL-producing Enterobacteriaceae (ESBL-E) are mostly associated with urinary tract infections, but may also cause significant bloodstream infections, which result in increased hospital costs, length of stay and patient mortality.3,4 Therefore, optimal detection methods for these ESBLs are becoming a major health issue.

MICs of expanded-spectrum cephalosporins (ESCs) for ESBL-E may be increased only slightly as compared with non-ESBL-E, thus leading to misidentification.5,6 A series of easy to carry out tests, mostly based on synergy between clavulanic acid and ESCs, are recommended, but their main pitfall is limited sensitivity, especially with cephalosporinase-producing bacteria, and the requirement for an overnight culture and a further 18 h culture for detection of synergy.4,7 Detection of ESBLs at the genetic level is a valuable alternative to the phenotype-based methods, it is independent of gene expression and relatively rapid, as compared with susceptibility
testing and culture results.

Here we sought to develop a LightCycler real-time PCR (LC-PCR) assay based on hybridization probes to detect CTX-M producers and to differentiate alleles belonging to the five phylogenetic groups directly from urine samples. Using this technique CTX-M-positive urine samples were accurately identified.

Materials and methods

Urine samples and microscopic examination

Over a 3 month period (15 November 2005 to 15 February 2006) urine samples that were positive for GNB were collected. An aliquot of 20 mL from each urine was immediately frozen to −20°C. Gram staining and microscopic reading were done systematically by examining 50 fields. The shapes and number of microorganisms and cells per oil immersion field were recorded. The presence of ≥1 microorganism uniformly distributed per field, after observation of at least 20 fields, was considered as positive for >10³ GNB/mL.

Bacterial identification and disc diffusion susceptibility testing were performed as described previously. Roughly 80% of GNB isolates were Enterobacteriaceae, mainly 

E. coli

Klebsiella oxytoca

Phylogenetic identification

Nucleic acid extractions, standard PCR and DNA sequencing

Whole-cell DNAs were extracted either from standard strains using the QIAamp DNA Mini Kit (Qiagen, Les Ulis, France) or from 106 urine samples positive for GNB according to Gram staining (36 ESBL-producing and 70 non-ESBL-producing Gram-negative isolates) using the QIAamp Viral RNA Mini Kit (Qiagen). PCR of the entire blaCTX-M-like, blaTEM-like and blaSHV-like genes, sequencing and sequence analysis were as previously described.

Construction of the internal amplification control (IAC)

An IAC was constructed as described previously to monitor potential PCR inhibitors and ensure successful amplification. The IAC, a 400 bp PCR fragment derived from pUC19 plasmid DNA, was used at 10⁴ copies and was detected using two detection primers, pUC19-3i-fluo and pUC19-5i-Red610 (Table 1). This control probes were used at 10⁴ copies and was detected using two detection primers, pUC19-3i-fluo and pUC19-5i-Red610 (Table 1).

LC-PCR

PCR primers CTX-M-A1 and CTX-M-A6 were designed to amplify a 444 bp fragment from all the blaCTX-M genes [Table 1 and Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. All LC-PCRs were performed using the LightCycler system 2.0 (Roche Diagnostics, Meylan, France) in a final volume of 20 uL.

The LightCycler-FastStart DNA Master plus SYBR Green I Kit (Roche Diagnostics) was used for SYBR Green detection, with the following amplification protocol: 94°C for 10 min; and 40 cycles consisting of 94°C for 10 s, 57°C for 10 s and 72°C for 10 s, followed by a melting curve analysis. Amplification of products was monitored at 530 nm.

The amplification mixture for LC-PCR with specific product detection (HYB-probe LC-PCR) consisted of 4 uL of reaction mixture (FastStart master hybridization probes; Roche Diagnostics), 3 mM MgCl₂, 0.8 uM of each amplification primer (Table 1), 0.3 uM of each anchor and sensor probe (Table 1 and Figure S1), 5 uL of template DNA and 2 uL of the IAC in a final volume of 20 uL. Samples were amplified as follows: 95°C for 10 min; and 40 cycles of 95°C for 10 s, 50°C for 10 s and 72°C for 22 s. Positive samples were identified by the instrument at the cycle number (Ct) where the fluorescence rose above background. A melting curve analysis was then performed as recommended by the manufacturer. Samples with amplicons having melting temperatures at the correct temperature were scored as positive for the respective target genes.

Sensitivity of LC-PCR assays for CTX-M detection

To assess the sensitivity of the LC-PCR assays for detection of the blaCTX-M genes in urine samples, overnight cultures of five CTX-M-producing Enterobacteriaceae were diluted in ESBL-negative urine samples to yield final spiking levels of 10⁵–10⁶ cfu/mL. DNA extracted from each dilution was tested by HYB-probe LC-PCR assay.

Results and discussion

ESBL-producing GNB (ESBL-GNB) in urine samples

Among the 5500 urine samples that were sent to the laboratory over the study period, 810 (from 655 different patients) were retained as being positive for GNB according to Gram staining results (e.g. containing at least 10³ GNB/mL). Thirty-six (from 29 patients) contained ESBL-GNB according to synergy testing, thus yielding a prevalence of ESBL-GNB in urine samples of 4.4% (or 4.2% per patient) [Table 2 and Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. ESBL prevalence in the urine samples increased by a factor of 3 in the last 5 years at Bicêtre hospital, similar to what has been observed in other hospitals/countries (Table 2).

The ESBL-producing bacterial species were: E. coli (22/29, 77%); Klebsiella pneumoniae (2/29, 7%); Citrobacter freundii (2/29, 7%); Providencia stuartii (1/29, 3%); Enterobacter cloacae (1/29, 3%); and Enterobacter aerogenes (1/29, 3%). These isolates were also resistant to other non-β-lactam antibiotics used for treating urinary tract infections such as gentamicin (60%), co-trimoxazole (70%) and ciprofloxacin (90%), but remained susceptible to carbapenems (imipenem, ertapenem and meropenem). These isolates were mostly from patients in the nephrology (27%), emergency (24%), gerontology (17%) and urology (10%) departments. For 52% of the patients, a urinary tract infection was diagnosed upon admission to the hospital, most of the patients were elderly people (median age 68 years) and the sex ratio was 17 females to 12 males (Table S1).
Oxacelay et al.

Table 1. Amplification, anchor and sensor primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Position</th>
<th>Comments</th>
<th>Sequence 5’ to 3’a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M-A1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>208–227&lt;sup&gt;c&lt;/sup&gt;</td>
<td>forward amplification primer</td>
<td>SCVATGTGCAGYACCAGTAA</td>
</tr>
<tr>
<td>CTX-M-A6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>629–648&lt;sup&gt;c&lt;/sup&gt;</td>
<td>forward amplification primer</td>
<td>TGGTRAYRTGGMTBAARGGCA</td>
</tr>
<tr>
<td>CTX-M-1-Anc</td>
<td>434–452&lt;sup&gt;c&lt;/sup&gt;</td>
<td>anchor primer for CTX-M-1</td>
<td>CCGGCCTACATACACCGATA-fluo</td>
</tr>
<tr>
<td>CTX-M-1-sen</td>
<td>455–471&lt;sup&gt;c&lt;/sup&gt;</td>
<td>detection primer for CTX-M-1</td>
<td>Red705-GTGGCGATGAAATGAGCTAT-flucose</td>
</tr>
<tr>
<td>CTX-M-2-Anc</td>
<td>325–340&lt;sup&gt;c&lt;/sup&gt;</td>
<td>anchor primer for CTX-M-2</td>
<td>ACAATCCCATTTGGAGAA-fluo</td>
</tr>
<tr>
<td>CTX-M-9-Anc</td>
<td>323–341&lt;sup&gt;c&lt;/sup&gt;</td>
<td>anchor primer for CTX-M-9</td>
<td>TTAATGACACCGATTTGCGAAAA-fluo</td>
</tr>
<tr>
<td>CTX-M-9-2-sen</td>
<td>344–363&lt;sup&gt;c&lt;/sup&gt;</td>
<td>detection primer for CTX-M-9 and CTX-M-2</td>
<td>Red640-ACGTCACCCACGACGAGAG-fluo</td>
</tr>
<tr>
<td>CTX-M-9-2-5 Anc</td>
<td>529–548&lt;sup&gt;c&lt;/sup&gt;</td>
<td>anchor primer for CTX-M-8 and CTX-M-25</td>
<td>CCGGCACCCCGCGCATAC-fluo</td>
</tr>
<tr>
<td>CTX-M-8-25-sen</td>
<td>552–570&lt;sup&gt;c&lt;/sup&gt;</td>
<td>detection primer for CTX-M-8 and CTX-M-25</td>
<td>Red670-CACGCAATTGCGATGCG-fluo</td>
</tr>
<tr>
<td>pUC19-3-fluo</td>
<td>330–350&lt;sup&gt;d&lt;/sup&gt;</td>
<td>anchor primer for pUC19</td>
<td>GAAAGCAAGTTAAAGTGTTGAAAC-fluo</td>
</tr>
<tr>
<td>pUC19-5i-Red610</td>
<td>350–375&lt;sup&gt;d&lt;/sup&gt;</td>
<td>detection primer for pUC19</td>
<td>Red610-CAGGGTTTCCCCGATCGAC-phosphate</td>
</tr>
<tr>
<td>pUC19fw</td>
<td>49–65&lt;sup&gt;d&lt;/sup&gt;</td>
<td>amplification primer pUC19</td>
<td>CCGAGACCCCTACAGCT</td>
</tr>
<tr>
<td>pUC19rv</td>
<td>433–448&lt;sup&gt;d&lt;/sup&gt;</td>
<td>amplification primer pUC19</td>
<td>TTGCATGCTGCAAGT</td>
</tr>
<tr>
<td>CTX-M-A1</td>
<td>208–227&lt;sup&gt;e&lt;/sup&gt;</td>
<td>PCR amplification primer</td>
<td>SCVATGTGCAGYACCAGTAA</td>
</tr>
<tr>
<td>CTX-M-9-2-5 Anc</td>
<td>950–931&lt;sup&gt;f&lt;/sup&gt;</td>
<td>PCR amplification primer CTX-M-1 group</td>
<td>CCGGTTTCCGGTATCAGAC-fluo</td>
</tr>
<tr>
<td>Pre-CTX-M-3b</td>
<td>950–931&lt;sup&gt;f&lt;/sup&gt;</td>
<td>PCR amplification primer CTX-M-1 group</td>
<td>CTGCGGGTTTCCGGTATCAGAC-fluo</td>
</tr>
<tr>
<td>iSECp-Prom+</td>
<td>6218–6236&lt;sup&gt;c&lt;/sup&gt;</td>
<td>PCR amplification primer CTX-M-1 group</td>
<td>CTGCGGGTTTCCGGTATCAGAC-fluo</td>
</tr>
<tr>
<td>IS903-Bint</td>
<td>7805–7785&lt;sup&gt;c&lt;/sup&gt;</td>
<td>PCR amplification primer CTX-M-9 group</td>
<td>CTGCGGGTTTCCGGTATCAGAC-fluo</td>
</tr>
</tbody>
</table>

<sup>a</sup>Hybridization probes were labelled with fluorescein, LC Red610, LC Red640, LC Red670 or LC Red705. M, A or C; W, A or T; R, A or G; Y, C or T; B, C, G or T; S, C or G; V, G, A or C.

<sup>b</sup>HPLC-purified oligonucleotides were used for real-time PCR amplification (Sigma Proligo, St-Quentin Fallavier, France).

<sup>c</sup>As compared with the sequence of CTX-M-3, GenBank Y10278.

<sup>d</sup>As compared with the sequence GenBank AF458080.

<sup>e</sup>As compared with the sequence GenBank X92506.

Table 2. Prevalence of ESBLs in urine samples at Bicêtre hospital from 2001 to 2006

<table>
<thead>
<tr>
<th>Study period</th>
<th>15/11/01–15/02/02</th>
<th>15/11/02–15/02/03</th>
<th>15/11/03–15/02/04</th>
<th>15/11/04–15/02/05</th>
<th>15/11/05–15/02/06</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNB (n)</td>
<td>949</td>
<td>867</td>
<td>959</td>
<td>925</td>
<td>810</td>
</tr>
<tr>
<td>Enterobacteriaceae (n)</td>
<td>827</td>
<td>785</td>
<td>868</td>
<td>837</td>
<td>720</td>
</tr>
<tr>
<td>ESBL (n)</td>
<td>12</td>
<td>22</td>
<td>29</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>ESBL-GNB (%)</td>
<td>1.3</td>
<td>2.5</td>
<td>3</td>
<td>2.7</td>
<td>4.4</td>
</tr>
<tr>
<td>ESBL-E (%)</td>
<td>1.5</td>
<td>2.8</td>
<td>3.3</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

**LC-PCR detection of bla<sub>CTX-M</sub> genes in control strains**

Fluorescence monitoring of product accumulation and detection by SYBR Green was used to test the amplification primers CTX-M-A1 and CTX-M-A6 on DNA extracted from pure CTX-M reference strains. Monitoring by gel electrophoresis revealed a single amplification product of high intensity and of the expected size (444 bp). No accumulation was observed with the negative controls, i.e. E. coli DH10B and Tris-EDTA solution (data not shown).

Similar results were obtained with HYB-probe LC-PCR hybridization probes, and the subsequent melting curve analysis monitored at the various wavelengths revealed single peaks for each bla<sub>CTX-M-type</sub> gene: bla<sub>CTX-M-1/3/5</sub> at 705 nm; bla<sub>CTX-M-8</sub> at 675 nm; bla<sub>CTX-M-2</sub> at 495 nm; and bla<sub>CTX-M-25</sub> at 580 nm. HYB-probe detection was highly specific; since only CTX-M producers were detected and no cross-reactivity could be observed with K. oxytoca strains that produce a structurally related β-lactamase (KOXY). Similarly, no signal was obtained with E. coli isolates or Tris-EDTA solution as negative controls.

**LC-PCR detection of CTX-M producers in urine samples**

Detection limits of HYB-probe LC-PCR using the different hybridization probes on bacterial DNA extracted from artificially spiked urine samples were 10<sup>2</sup>–10<sup>3</sup> bacteria/mL of urine. Samples were run in triplicate, and the Ct values obtained in three different runs indicated that the LC-PCR assay was highly reproducible. Negative results were always validated by amplification of the IAC. Among the 70 ESBL-negative isolates, none gave positive PCR results.

HYB-probe LC-PCR detected in 25 out of the 36 ESBL-positive urines bla<sub>CTX-M</sub> genes that belonged to three of the five CTX-M clusters, i.e. 17 to CTX-M-1, 6 to CTX-M-9 and...
CTX-M ESBLs in urine samples

2 to CTX-M-2. For a few patients, with repeated urine samples, reproducible identification of the same CTX-M type was achieved.

Standard PCR followed by sequencing confirmed the HYB-probe LC-PCR results. Three different types of CTX-M enzymes were detected: CTX-M-15, n=17; CTX-M-14, n=6; and CTX-M-2, n=2. In this study, CTX-M enzymes represented 66% (19/29) of the ESBLs and CTX-M-15 (13/19, 70%) was the predominant CTX-M-type β-lactamase. This situation mirrors the current trend observed in many hospitals worldwide.1,2 The remaining 11 non-CTX-M ESBLs were 8 TEM types (TEM-3, TEM-21 and TEM-24) and 3 SHV types (SHV-2 and SHV-12) (Table S1).

Conclusions

Here, we show the technical feasibility of an LC-PCR detection assay used directly on urine samples. This technique is based on hybridization probes, conferring high specificity and sensitivity. Indeed, no false-positive results were obtained, and all the CTX-M-positive urine samples were detected repeatedly and CTX-M groups could be identified unambiguously. In the current epidemiological situation, one-third of the ESBLs were missed using this technique since TEM and SHV ESBLs were not detected. Thus, this assay should be coupled with another detection method geared towards TEM and SHV enzymes. Thus most if not all ESBLs would be detected.

The ease, speed and reliability of the LC-PCR technique makes it a powerful technique for detection of CTX-M producers. It may be used to control the emerging blaCTX-M resistance determinants, which are becoming a major public health issue.

Acknowledgements

We thank C. Spicq for assistance in database computing, and F. Lartigue and N. Fortineau for helpful discussions. This work was presented in part at the Seventeenth European Congress of Clinical Microbiology and Infectious Diseases, Munich, Germany, 2007 (poster/abstract number P572).

Funding

This work was funded by a grant from the Ministère de l’Education Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, Paris, by the Assistance Publique-Hôpitaux de Paris, France, by the European Community (6th PCRD, LSHMCT- 2003-503-335, TROCAR contract HEALTH-F3-2008-223031) and by INSERM, France.

Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References