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

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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 bla$_{CTX-M}$-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 bla$_{CTX-M}$ genes using the LC-PCR assay. The bla$_{CTX-M}$ genes belonged to the bla$_{CTX-M-1}$, bla$_{CTX-M-9}$ and bla$_{CTX-M-2}$ groups (68%, 24% and 8%, respectively). Standard PCR and sequencing of the entire bla$_{CTX-M}$ 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. Several molecular techniques for TEM, SHV, GES-type and CTX-M ESBL detection have been described, and so far evaluated only on pure cultures. 

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 sample was immediately frozen to −20°C. Gram staining and microscopic reading were done systemically by examining 50 fields. The shapes and number of microorganisms and cells per oil immersion were recorded. The presence of ≥1 microorganism uniformly distributed per field, after observation of at least 20 fields, was considered as positive for >10² GNB/mL. Leucocytes were manually counted using Kova slides (Hycor Biomedicals, Pencuik, UK) and interpreted as recommended.

Bacterial strains and microbiological techniques

Ten CTX-M producers were used as positive controls. Eight Klebsiella oxytoca clinical isolates were also used as controls since they produce structurally related but chromosomally encoded ESBLs (KOXY/K1). Escherichia coli DH10B was used as a CTX-M-negative control.

Bacterial identification and disc diffusion susceptibility testing were performed as described previously. The presence of ESBLs was inferred by a synergy image using the double-disc synergy test with the CTX-M-negative control. 

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 bla_{CTX-M-like}, bla_{TEM-like} and bla_{SHV-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).

LC-PCR

PCR primers CTX-M-A1 and CTX-M-A6 were designed to amplify a 444 bp fragment from all the bla_{CTX-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 µL.

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 µL of reaction mixture (Fast-Start master hybridization probes; Roche Diagnostics), 3 mM MgCl₂, 0.8 µM of each amplification primer (Table 1), 0.3 µM of each anchor and sensor probe (Table 1 and Figure S1), 5 µL of template DNA and 2 µL of the IAC in a final volume of 20 µL. 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 bla_{CTX-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 10⁶–10⁷ 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 10⁴ 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).

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LC-PCR detection of \textit{bla}_{\text{CTX-M}} 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. \textit{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 \textit{bla}_{\text{CTX-M-1/3/15}}, \textit{bla}_{\text{CTX-M-1/3/15}} at 705 nm; \textit{bla}_{\text{CTX-M-2/8}}, 67.5°C at 670 nm; \textit{bla}_{\text{CTX-M-2/8}}, 49.5°C at 640 nm; \textit{bla}_{\text{CTX-M-2/8}}, 58°C at 670 nm; and \textit{bla}_{\text{CTX-M-2/8}}, 60°C at 640 nm. HYB-probe detection was highly specific, since only CTX-M producers were detected and no cross-reactivity could be observed with \textit{K. oxytoca} strains that produce a structurally related β-lactamase (KOXY). Similarly, no signal was obtained with \textit{E. coli} isolates or Tris-EDTA solution as negative controls.

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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Position</th>
<th>Comments</th>
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<tr>
<td>CTX-M-A1</td>
<td>208–227</td>
<td>forward amplification primer</td>
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<tr>
<td>CTX-M-A6</td>
<td>629–648</td>
<td>anchor primer for CTX-M-1</td>
</tr>
<tr>
<td>CTX-M-1-Anc</td>
<td>434–452</td>
<td>anchor primer for CTX-M-1</td>
</tr>
<tr>
<td>CTX-M-1-sen</td>
<td>455–471</td>
<td>detection primer for CTX-M-1</td>
</tr>
<tr>
<td>CTX-M-2-Anc</td>
<td>325–340</td>
<td>anchor primer for CTX-M-2</td>
</tr>
<tr>
<td>CTX-M-9-Anc</td>
<td>323–341</td>
<td>anchor primer for CTX-M-9</td>
</tr>
<tr>
<td>CTX-M-9-2-sen</td>
<td>344–363</td>
<td>detection primer for CTX-M-9 and CTX-M-2</td>
</tr>
<tr>
<td>CTX-M-8-25-Anc</td>
<td>529–548</td>
<td>anchor primer for CTX-M-8 and CTX-M-25</td>
</tr>
<tr>
<td>CTX-M-8-25-sen</td>
<td>552–570</td>
<td>detection primer for CTX-M-8 and CTX-M-25</td>
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<td>pUC19-3i-fluo</td>
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<td>anchor primer for pUC19</td>
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<tr>
<td>pUC19-3i-Red610</td>
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<td>pUC19fw</td>
<td>49–65</td>
<td>amplification primer pUC19</td>
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<tr>
<td>pUC19rv</td>
<td>433–448</td>
<td>amplification primer pUC19</td>
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<tr>
<td>CTX-M-A1</td>
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<td>CTX-M-A2</td>
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<td>pUC19rv</td>
<td>433–448</td>
<td>PCR amplification primer pUC19</td>
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<tr>
<td>Pre-CTX-M-3b</td>
<td>950–931</td>
<td>PCR amplification primer CTX-M-1 group</td>
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<tr>
<td>IScep-Prom+</td>
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<td>PCR amplification primer CTX-M-1 group</td>
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<tr>
<td>IS903-Bint</td>
<td>7805–7785</td>
<td>PCR amplification primer CTX-M-9 group</td>
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*aHybridization 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.*

**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>
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<tr>
<td>GNB (n)</td>
<td>949</td>
<td>867</td>
<td>959</td>
<td>925</td>
<td>810</td>
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<tr>
<td>Enterobacteriaceae (n)</td>
<td>827</td>
<td>785</td>
<td>868</td>
<td>837</td>
<td>720</td>
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<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>3.3</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 \textit{bla}_{\text{CTX-M}} genes in control strains**

Detection limits of HYB-probe LC-PCR using the different hybridization probes on bacterial DNA extracted from artificially spiked urine samples were $10^2$–$10^3$ 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.

**Summary**

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LC-PCR detection of \textit{bla}_{\text{CTX-M}} 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. \textit{E. coli} DH10B and Tris-EDTA solution (data not shown).

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HYB-probe LC-PCR detected in 25 out of the 36 ESBL-positive urines \textit{bla}_{\text{CTX-M}} genes that belonged to three of the five CTX-M clusters, i.e. 17 to CTX-M-1, 6 to CTX-M-9 and
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 \( \beta \)-lactamase. This situation mirrors the current trend observed in many hospitals worldwide.\(^1\)(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 \( \text{bla}_{\text{CTX-M}} \) resistance determinants, which are becoming a major public health issue.

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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