Short communication


Hiran Dhanji a,*, Michel Doumith a, Olivier Clermont b, Erick Denamur b, Russell Hope a, David M. Livermore a, Neil Woodford a

a Antibiotic Resistance Monitoring and Reference Laboratory, Centre for Infections, Health Protection Agency, London NW9 5EQ, UK
b INSERM U722 and Université Paris 7, Faculté de Médecine, Site Xavier Bichat, 75018 Paris, France

Article info
Article history:
Received 4 May 2010
Accepted 8 June 2010

Keywords:
Escherichia coli
Epidemic
LightCycler® PCR
ESBL
Pandemic clone

Abstract
CTX-M-15 has become the most prevalent extended-spectrum β-lactamase amongst Escherichia coli in many countries during the past decade. Its dominance partly reflects the dissemination of an E. coli O25b:H4 ST131 clone that commonly produces this enzyme. We describe rapid real-time polymerase chain reaction (PCR) assays able to detect E. coli belonging to the ST131 clone and to identify blaCTX-M-15-like.

© 2010 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.

1. Introduction

Many recent studies have highlighted the worldwide spread of an Escherichia coli O25b:H4 ST131 clone belonging to phylogenetic group B2 as a gut colonist and an agent of urinary infections. The clone is frequently multiresistant and commonly produces the CTX-M-15 extended-spectrum β-lactamase (ESBL). It constitutes a major public health concern [1–5] and several groups have described methods for its rapid detection and recognition [6–8]. In particular, Clermont et al. [6] developed a polymerase chain reaction (PCR) assay specific for the allelic variant of the pabB gene typical of the clone (allele 15) (http://www.pasteur.fr/recherche/genopole/PPB/mlst/Ecoli.html). However, like many allele-specific PCRs, it has the drawback that 3′-end degradation of the primers may cause false-positive results. The assay is based on detecting two single nucleotide polymorphisms (SNPs), namely thymine-144 and adenine-450, in pabB. This combination is unique to ST131 and the occurrence of both of these pabB SNPs implies that an isolate belongs to ST131.

Although the ST131 clone is most strongly associated with CTX-M-15 ESBL [5], it may also host other ESBLs or acquired AmpC enzymes and sometimes occurs as a quinolone-resistant type with no ESBL [4]. Against this background, it is desirable to have rapid specific methods for the rapid detection both of blaCTX-M-15 and the ST131 clone; thus, we sought to develop these methods.

2. Materials and methods

2.1. Primers for detection of the ST131 clone

Primer pairs designed to amplify 40-bp (Thymine ‘T’ SNP assay) and 49-bp (Adenine ‘A’ SNP assay) regions containing the ST131-specific changes within pabB were ST131TF (5′-GGT GCT CCA GCA GGT G-3′) with ST131TR (5′-TGG GCG AAT GTC TGC-3′), and ST131AF (5′-GGC AAT CCA ATA TGA CCC-3′) with ST131AR (5′-ACC TGG CGA AAT TTT TCG-3′), respectively.

2.2. Primers to distinguish blaCTX-M-15-like genes

Primers were designed to amplify a 49-bp region conserved amongst group 1 CTX-M genes as follows: MC-3-15F (5′-GGT GCT CCA GCA GGT G-3′) with ST131TR (5′-TGG GCG AAT GTC TGC-3′), and ST131AF (5′-GGC AAT CCA ATA TGA CCC-3′) with ST131AR (5′-ACC TGG CGA AAT TTT TCG-3′), respectively.

2.3. Real-time PCR cycling conditions

Real-time PCR amplification and melting curve analysis were performed using a LightCycler® with software version 3.5 (Roche Diagnostics, Chichester, UK).
The real-time PCR mixture was prepared using the LightCycler® FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) following the manufacturer’s protocol. Cycling conditions for the ST131 assays were: initial denaturation for 5 min at 95 °C and 40 cycles of 5 s at 95 °C and 10 s at 58 °C; those for the blacTX-M-15 assay were: 5 min at 95 °C and 20 cycles of 5 s at 95 °C and 10 s at 70 °C. The fluorescence signal was measured at the end of each annealing step. Following amplification, a melting curve was generated by heating the PCR product to 95 °C with a ramp rate of 0.05 °C/s.

2.4. Validation of real-time PCR assays for ST131 and blacTX-M-15-like

The ST131 assay was validated using 164 E. coli isolates. Eighty-four isolates were identified previously as belonging to the

![Real-time polymerase chain reaction (PCR) detection of (a, b) Escherichia coli clone ST131 and (b) the blacTX-M-15 allele by analysis of amplicon melt curves. (a) Thymine ‘T’ assay for pabB with primers ST131TF/ST131TR to identify T144 single nucleotide polymorphism (SNP): black peak (melting temperature ($T_m$) = 82.82 °C) = ST131 E. coli; grey dotted peak ($T_m$ = 84 °C) = non-ST131 E. coli. (b) Adenine ‘A’ assay for pabB with primers ST131AF/ST131AR to identify A450 SNP; black peak ($T_m$ = 80.69 °C) = ST131 E. coli; grey dotted peak ($T_m$ = 82 °C) = non-ST131 E. coli. (c) Group 1 blacTX-M assay with primers MC-3-15F/MC-3-15R; black peak ($T_m$ = 84 °C) with CTX-M-15-like gene; grey dotted peak ($T_m$ = 82.82 °C) with CTX-M-3-like gene.](image-url)
Table 1

<table>
<thead>
<tr>
<th>E. coli strains (n)</th>
<th>E. coli positive with ST131 Thymine ‘T’ SNP assay (Tm = 82.82 °C) [%]</th>
<th>E. coli positive with ST131 Adenine ‘A’ SNP assay (Tm = 80.69 °C) [%]</th>
<th>Isolates assigned ST131 based on ‘A’ and ‘T’ SNP assays [%]</th>
<th>E. coli positive with CTX-M-15-like assay (Tm = 84 °C) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST131 with blaCTX-M-3 (52)</td>
<td>52 (100%)</td>
<td>52 (100%)</td>
<td>52 (100%)</td>
<td>Negative</td>
</tr>
<tr>
<td>ST131 with blaCTX-M-15 (32)</td>
<td>32 (100%)</td>
<td>32 (100%)</td>
<td>32 (100%)</td>
<td>32 (100%)</td>
</tr>
<tr>
<td>blaCTX-M-15 non-ST131 (40)</td>
<td>1 (2.5%)</td>
<td>0</td>
<td>0</td>
<td>N/T</td>
</tr>
<tr>
<td>blaCTX-M-1 (1)</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>Negative</td>
</tr>
<tr>
<td>blaCTX-M-2 (1)</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>Negative</td>
</tr>
<tr>
<td>blaCTX-M-6 (1)</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>Negative</td>
</tr>
<tr>
<td>blaCTX-M-14 (1)</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>Negative</td>
</tr>
<tr>
<td>blaCTX-M-15 (1)</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>Negative</td>
</tr>
</tbody>
</table>

N/T, not tested.

* Only isolates positive in both the Thymine ‘T’ and Adenine ‘A’ SNP assays belong to the ST131 clone.

O25b-ST131 clone by an allele-specific PCR assay for the pabB gene [6], including 52 isolates with CTX-M-3 and 32 isolates with CTX-M-15/28 enzymes. The remaining 80 isolates were known not to belong to ST131: they included 10 of each phylogenetic group A, B1, B2 and D all with CTX-M-15 ESBL, and another 10 each of these phylogenetic groups lacking ESBLs. The pabB genes of the 80 non-ST131 isolates were sequenced using an ABI Genetic Analyser capillary platform 3130XL (Applied Biosystems, Foster City, CA) to confirm that they did not contain either of the ST131-specific pabB SNPs sought here.

The blaCTX-M-15-like assay was validated using the above-mentioned 84 O25b-ST131 isolates as well as 6 others with blaCTX-M-1, -2, -8, -9, -14 and -25. The blaCTX-M-2 gene was sequenced for 32 isolates with CTX-M-15/28 ESBLs to define the precise alleles present. Controls were representatives of pulsed-field gel electrophoresis (PFGE)-defined variants of the ST131 clone, namely UK strains A (CTX-M-15; NCTC 13441) and C (CTX-M-3; NCTC 13452) [9].

3. Results

3.1. ST131 assays

All of the isolates (n = 84) previously identified as belonging to the ST131 clone by allele-specific PCR assay [6] produced PCR products with a mean ± standard deviation (S.D.) Tm value of 82.82 ± 0.08 °C and 80.69 ± 0.09 °C in the Thymine ‘T’ and Adenine ‘A’ SNP real-time PCR assays, respectively (Tables 1 and 2; Fig. 1a and b). The standard error of the mean (S.E.M.) for the Adenine ‘A’ and Thymine ‘T’ SNP real-time PCR assays was 0.01 °C. Amongst the controls, 5 of 80 non-ST131 isolates produced a product with a Tm of 82.82 ± 0.08 °C in the Thymine ‘T’ SNP assay, whereas none gave a Tm of 80.69 ± 0.09 °C in the Adenine ‘A’ SNP assay (Table 1). Sequencing revealed that these five isolates had the T144 SNP but not the A450 SNP; one had CTX-M-15 ESBL and belonged to phylogenetic group D, whilst the other four false-positive isolates belonged to phylogenetic group B2 and lacked ESBLs (Table 1).

Based on the findings of Clermont et al. [6], only isolates that contained both T144 and A450 SNPs were assigned to ST131. Hence, the sensitivity and specificity values were 100% because all 84 isolates known to belong to ST131 were positive in both ‘A’ and ‘T’ SNP assays, whereas all 80 non-ST131 isolates were negative for at least one of the two SNPs.

3.2. blaCTX-M-15-like assay

The 32 isolates with previously sequenced blaCTX-M-15 produced identical peaks with a mean ± S.D. Tm of 84 ± 0.19 °C, exactly the same as UK strain A in the real-time blaCTX-M-15-like assay (Tables 1 and 2; Fig. 1c). The S.E.M. was 0.03 °C. No peaks were obtained in the assay for isolates harbouring non-group 1 CTX-M-ESBLs, specifically blaCTX-M-2, -8, -9, -14 and -25, whereas Tm peaks distinct from that for blaCTX-M-15 were obtained for the 53 isolates harbouring the group 1 genes blaCTX-M-3 (n = 52) and blaCTX-M-1 (n = 1). These latter 53 isolates produced identical Tm peaks at 82.82 °C to that obtained with a representative isolate of UK strain C, known to harbour CTX-M-3 enzyme [9]. The blaCTX-M-15-like assay had 100% sensitivity and specificity because only isolates (n = 32) shown to produce CTX-M-15 gave a positive result in the assay, whilst all isolates (n = 58) not producing CTX-M-15-like ESBLs were negative.

4. Discussion

Here we describe three novel real-time PCR assays. Used together the ‘T’ and ‘A’ ST131-specific pabB SNP assays rapidly and accurately detected all E. coli isolates that belonged to the ST131 clone. Unlike the earlier method of Clermont et al. [6], which depends on detecting the same SNPs, our assay is not affected by 3’-end primer degradation that may otherwise cause false-positive results. Using pabB to identify ST131 isolates is based on the detection of A450 and T144 SNPs that are found together only in ST131 [6]. Hence, both our ‘A’ and ‘T’ SNP assays must be performed and only isolates positive in both assays should be assigned to ST131. Consistent with this, five non-ST131 E. coli isolates that were positive in the ‘T’ SNP assay were negative in the ‘A’ assay. The most cost-effective approach may be to perform the ‘T’ SNP assay only on those isolates first giving positive results in the ‘A’ assay. There was very little Tm variation from the mean in both the ‘A’ and ‘T’ SNP assays (mean ± S.D. 80.69 ± 0.09 °C and 82.82 ± 0.08 °C, respectively), and the standard error for both assays was also very small (0.01 °C). When used together, these assays are able to assign precisely E. coli to ST131 based on melting curve analysis.

Our third assay accurately identified isolates harbouring the blaCTX-M-15-like ESBL genes and distinguished them from those with
other group 1 or with non-group 1 CTX-M ESBL genes. The primers used for amplification were specific for group 1 CTX-M ESBL and the assay detected an A/G SNP at position 725, which raised the Tm. Although some other CTX-M genes share G725, these are far rarer than CTX-M-15 types, which is the globally dominant ESBL. *Escherichia coli* producing CTX-M-1 and -3 ESBLs, the other common group 1 types, produced distinct Tm peaks from CTX-M-15.

The blacTX-M-15-like assay had 100% sensitivity and specificity and there was very little Tm variation from the mean (84 ± 0.19 °C) and the S.E.M. was also very small (0.03 °C), therefore the assay was able to distinguish precisely CTX-M-15-like ESBLs genes from other group 1 CTX-M ESBL genes based on melting curve analysis.

In the real-time PCR assays, SYBR Green I, a highly specific fluorescent DNA intercalating dye that detects PCR product as it accumulates during PCR cycles, was used. One problem is that SYBR Green I can generate false-positive signals because it binds to any double-stranded DNA, therefore melting curve analysis is critical for the real-time PCR methods described here; moreover the CTX-M-15 and ST131 assays cannot be multiplexed.

In summary, we have described two rapid and simple real-time PCR methods that are able: (i) to distinguish CTX-M-15-like ESBL genes from other group 1 and non-group 1 CTX-M-ESBL genes; and (ii) to detect *Escherichia coli* belonging to the internationally disseminated O25b:H4 ST131 clone. These assays should be useful rapid tools for screening *E. coli* isolates to elucidate transmission pathways of the ST131 clone and its predominant ESBL amongst community and hospital patients, animals, food and environmental sources.

**Funding:** No funding sources.

**Competing interests:** None declared.

**Ethical approval:** Not required.

**References**


