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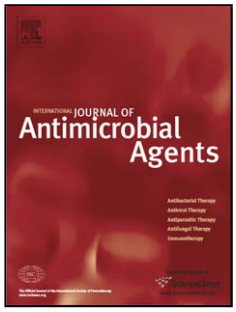

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Real-time PCR for detection of the O25b-ST131 clone of *Escherichia coli* and its CTX-M-15-like extended-spectrum β-lactamases

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ABSTRACT

CTX-M-15 has become the most prevalent extended-spectrum β-lactamase among *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 $bla_{\text{CTX-M-15-like}}$.

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/PF8/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 $bla_{\text{CTX-M-15}}$ 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 bla$_{CTX\text{-}M\text{-}15}$-like genes

Primers were designed to amplify a 49-bp region conserved among group 1 CTX-M genes as follows: MC-3-15F (5'-TGG GGG ATA AAA CCG GCA G-3') and MC-3-15R (5'-GCG ATA TCG TTG GTG GTG C-3'). The amplicon included the SNP at position 725 where *bla*$_{\text{CTX-M-15, -28, -29, -32, -52, -57, -62, -71, -79}}$ and -$_{89}$ have guanine whereas other group 1 genes have adenine, leading to a higher melting temperature (T_m) in the former case.

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 *bla*$_{\text{CTX-M-15}}$ 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 bla$_{\textit{CTX-M-15}}$*-like*

The ST131 assay was validated using 164 *E. coli* isolates. Eighty-four isolates were identified previously as belonging to the 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 of 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 *bla*$_{\text{CTX-M-15}}$-like assay was validated using the abovementioned 84 O25b-ST131 isolates as well as 6 others with *bla*$_{\text{CTX-M-1, -2, -8, -9, -14}}$ and -$_{25}$. The *bla*$_{\text{CTX-M}}$ 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.) T_m value of 83 ± 0.08 °C and 81 ± 0.09 °C in the Thymine 'T' and Adenine 'A' SNP real-time PCR assays, respectively (Tables 1 and 2; Fig. 1a,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. Among the controls, 5 of 80 non-ST131 isolates gave a product with a T_m of 83 ± 0.08 °C in the Thymine 'T' SNP assay, whereas none gave a T_m of 81 ± 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. bla$_{CTX-M-15}$-like assay

The 32 isolates with previously sequenced *bla*$_{CTX-M-15}$ produced identical peaks with a mean ± S.D. T_m of 84 ± 0.19 °C, exactly the same as UK strain A in the real-time *bla*$_{CTX-M-15}$-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 *bla*$_{CTX-M-2, -8, -9, -14}$ and $_{-25}$, whereas T_m peaks distinct from that for *bla*$_{CTX-M-15}$ were obtained for the 53 isolates harbouring the group 1 genes *bla*$_{CTX-M-3}$ (*n* = 52) and *bla*$_{CTX-M-1}$ (*n* = 1). These latter 53 isolates produced identical T_m peaks at 83 °C to that obtained with a representative isolate of UK strain C, known to harbour CTX-M-3 enzyme [9]. The *bla*$_{CTX-M-15}$-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 T_m variation from the mean in both the 'A' and 'T' SNP assays (mean ± S.D. 81 ± 0.09 °C and 83 ± 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 *bla*$_{\text{CTX-M-15}}$-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 T_m. 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 T_m peaks from CTX-M-15.

The *bla*$_{\text{CTX-M-15}}$-like assay had 100% sensitivity and specificity and there was very little T_m 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 *E. 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 among community and hospital patients, animals, food and environmental sources.

Funding

None.

Competing interests

None declared.

Ethical approval

Not required.

References

[1] Cerquetti M, Giufrè M, García-Fernández A, Accogli M, Fortini D, Luzzi I, et al. Ciprofloxacin-resistant, CTX-M-15-producing *Escherichia coli* ST131 clone in extraintestinal infections in Italy. Clin Microbiol Infect 2010 Jan 27 [Epub ahead of print].

[2] Johnson JR, Johnston B, Clabots C, Kuskowski MA, Pendyala S, Debroy C, et al. *Escherichia coli* sequence type ST131 as an emerging fluoroquinolone-resistant uropathogen among renal transplant recipients. Antimicrob Agents Chemother 2010;54:546–50.

[3] Leflon-Guibout V, Blanco J, Amaqdouf K, Mora A, Guize L, Nicolas-Chanoine MH. Absence of CTX-M enzymes but high prevalence of clones, including clone ST131, among fecal *Escherichia coli* isolates from healthy subjects living in the area of Paris, France. J Clin Microbiol 2008;46:3900–5.

[4] Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V, Demarty R, Alonso MP, Canica MM, et al. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. J Antimicrob Chemother 2008;61:273–81.

[5] Peirano G, Pitout JD. Molecular epidemiology of *Escherichia coli* producing CTX-M β-lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents 2010;35:316–21.

[6] Clermont O, Dhanji H, Upton M, Gibreel T, Fox A, Boyd D, et al. Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. J Antimicrob Chemother 2009;64:274–7.

[7] Lau SH, Cheesborough J, Kaufmann ME, Woodford N, Dodgson AR, Dodgson KJ, et al. Rapid identification of uropathogenic *Escherichia coli* of the O25:H4-ST131 clonal lineage using the DiversiLab repetitive sequence-based PCR system. Clin Microbiol Infect 2010;16:232–7.

[8] Pitout JD, Campbell L, Church DL, Wang PW, Guttman DS, Gregson DB. Using a commercial DiversiLab semiautomated repetitive sequence-based PCR typing technique for identification of *Escherichia coli* clone ST131 producing CTX-M-15. J Clin Microbiol 2009;47:1212–5.

[9] Woodford N, Carattoli A, Karisik E, Underwood A, Ellington MJ, Livermore DM. Complete nucleotide sequences of plasmids pEK204, pEK499 and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. Antimicrob Agents Chemother 2009;53:4472–82

Fig. 1. Real-time polymerase chain reaction (PCR) detection of (a,b) *Escherichia coli* clone ST131 and (b) the $bla_{\text{CTX-M-15}}$ 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) = 83 °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 = 81 °C) = ST131 *E. coli*; grey dotted peak (T_m = 82 °C) = non-ST131 *E. coli*. (c) Group 1 $bla_{\text{CTX-M}}$ 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 = 83 °C) with CTX-M-3-like gene.

Table 1

Escherichia coli isolates positive with the ST131 real-time assays and the CTX-M-15-like real-time assay

E. coli strains (*n*)	*E. coli* positive with ST131 Thymine 'T' SNP assay (T_m = 83 °C) [*n* (%)] [a]	*E. coli* positive with ST131 Adenine 'A' SNP assay (T_m 81 °C) [*n* (%)] [a]	Isolates assigned ST131 based on 'A' and 'T' SNP assays [*n* (%)]	*E. coli* positive with CTX-M-15-like assay (T_m = 84 °C) [*n* (%)]
ST131 with *bla*$_{CTX-M-3}$ (52)	52 (100%)	52 (100%)	52 (100%)	Negative
ST131 with *bla*$_{CTX-M-15}$ (32)	32 (100%)	32 (100%)	32 (100%)	32 (100%)
bla$_{CTX-M-15}$ non-ST131 (40)	1 (2.5%)	0	0	N/T
Non-ESBL/non-ST131 (40)	4 (10%)	0	0	N/T
bla$_{CTX-M-1}$ (1)	N/T	N/T	N/T	Negative
bla$_{CTX-M-2}$ (1)	N/T	N/T	N/T	Negative
bla$_{CTX-M-8}$ (1)	N/T	N/T	N/T	Negative
bla$_{CTX-M-9}$ (1)	N/T	N/T	N/T	Negative
bla$_{CTX-M-14}$ (1)	N/T	N/T	N/T	Negative

bla$_{CTX-M-25}$ (1)	N/T	N/T	N/T	Negative

N/T, not tested.

[a] Only isolates positive in both the Thymine 'T' and Adenine 'A' SNP assays belong to the ST131 clone.

Table 2

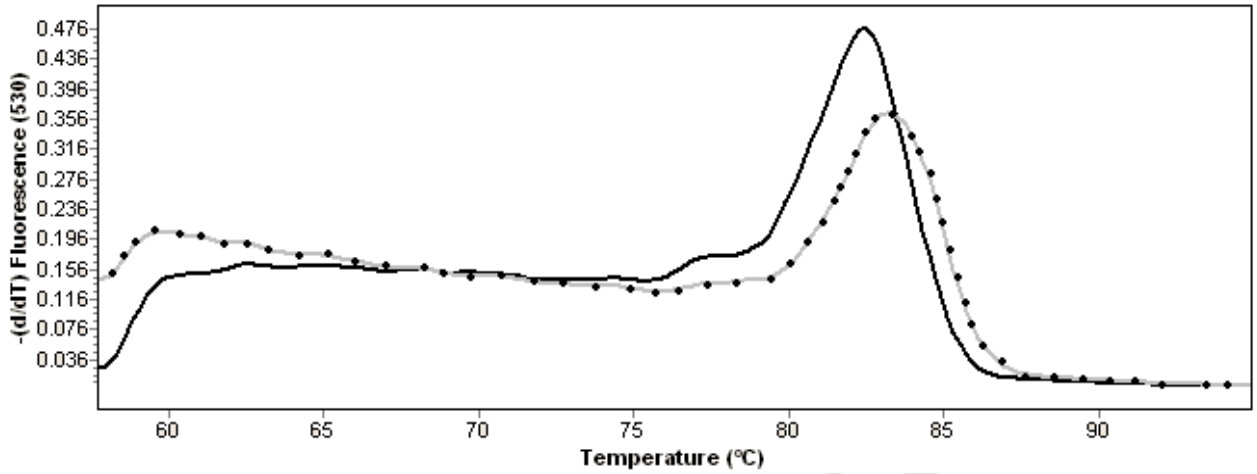
Summary statistics of the three real-time polymerase chain reaction (PCR) assays with respect to melting temperature (T_m) [a]

Statistic	Thymine 'T' SNP assay (n = 84)	Adenine 'A' SNP assay (n = 84)	CTX-M-15-like assay (n = 32)
Mean T_m (°C)	83	81	84
S.E. (°C)	0.01	0.01	0.03
S.D. (°C)	0.08	0.09	0.19

S.E., standard error; S.D., standard deviation.

[a] Data are calculated with respect to the T_m obtained from the 84 isolates known to be ST131 for the Thymine 'T' and Adenine 'A' assays (used to validate the ST131 assay) as well as 32 isolates known to produce CTX-M-15 (used to validate the CTX-M-15-like assay).

(a)



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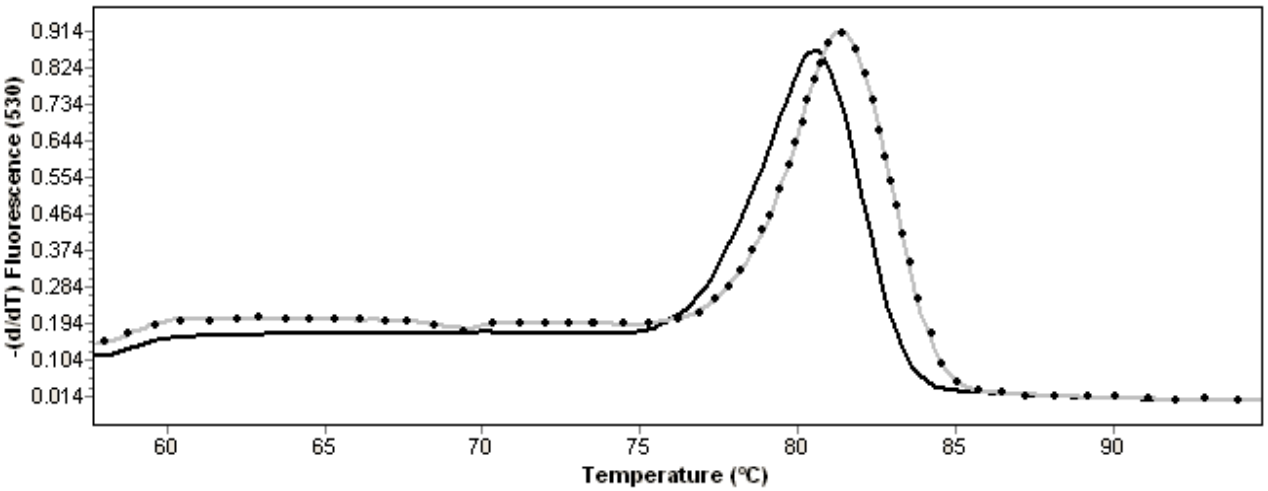

(c)