Rapid and simple detection of \textit{bla}_{CTX-M} genes by multiplex PCR assay

Li Xu,$^{1,2}$ Vicki Ensor,$^2$ Savita Gossain,$^1$ Kathy Nye$^1$ and Peter Hawkey$^{1,2}$

$^1$Health Protection Agency West Midlands Public Health Laboratory, Birmingham Heartlands & Solihull NHS Trust, Birmingham B9 5SS, UK
$^2$Division of Immunity and Infection, University of Birmingham, UK

A novel multiplex PCR assay is described (CTX-Mplex PCR) that allows rapid detection of \textit{bla}_{CTX-M} genes and discrimination between groups 1, 2, 9 and 25/26. The specificity and sensitivity of the assay were evaluated with 10 control strains and then applied to 62 clinical isolates. The multiplex PCR detected and classified \textit{bla}_{CTX-M} genes with 100% accuracy. The utilization of a denaturing HPLC WAVE system to size the PCR products automatically from the multiplex PCR enhances the assay by saving time and costs.

INTRODUCTION

Plasmid-mediated extended-spectrum $\beta$-lactamases (ESBLs) are the predominant cause of transferable resistance to third-generation cephalosporins in Gram-negative bacteria. CTX-M-type ESBLs, which are non-TEM and non-SHV derivatives, represent a new and rapidly growing family of molecular class-A ESBLs (Bonnet, 2004). On the basis of their amino acid sequence similarities, they have been classified into five groups, groups 1, 2, 8, 9 and 25/26, and, to date, more than 40 CTX-M $\beta$-lactamas have been reported (Bonnet, 2004).

In the past 15 years, CTX-M-type ESBLs have become more prevalent worldwide (Bonnet et al., 2000; Chanawong et al., 2002; Moland et al., 2003; Pagani et al., 2003). In the UK, the first report was of a CTX-M-9-producing isolate (group 9) of \textit{Klebsiella oxytoca} in Leeds in 2000 (Alobwede et al., 2003); subsequently, CTX-M-26 (group 25/26) was recovered from an outbreak in City Hospital, Birmingham (Brenwald et al., 2003). CTX-M-9, -14 (group 9) and -15-producing isolates (group 1) were found in both the community and hospitals in York (Munday et al., 2004). A more recent surveillance study from 42 centres showed that CTX-M-15-producing \textit{Escherichia coli} were widely scattered throughout the UK (Woodford et al., 2004). In addition, the first isolation of CTX-M-3 (group 1) $\beta$-lactamase has also been reported (Winstanley et al., 2004).

The diversity and increasing prevalence of CTX-M-type ESBLs pose a serious threat to the clinical use of third-generation cephalosporins for the treatment of severe infections. What is more alarming is the underdetection of ESBL production in clinical isolates, as it is common practice to screen for ceftazidime resistance as an indicator of ESBLs (Alobwede et al., 2003). However, in contrast with TEM- and SHV-type ESBLs, most CTX-M producers preferentially hydrolyse and confer resistance to cefotaxime rather than ceftazidime. There is an urgent need to use molecular detection methods that enable the identification and monitoring of the emergence of CTX-M-type ESBLs. Those methods which make possible the differentiation of different gene sequences to establish the epidemiology of these new ESBLs are particularly needed. PCR has been applied successfully to characterize \textit{bla}_{CTX-M} genes, but detection of all members from five groups required multiple PCRs with group-specific primers (Canton et al., 2002; Chanawong et al., 2002; Pitout et al., 2004) or consensus primers which only amplify few \textit{bla}_{CTX-M} alleles (Cao et al., 2002; Saladin et al., 2002). Currently, sequencing is the only method for definitive identification of \textit{bla}_{CTX-M} genes, which is labour-intensive, time-consuming and expensive. We report here the development of a rapid and accurate multiplex PCR assay (CTX-Mplex PCR) for the simultaneous amplification of all \textit{bla}_{CTX-M} genes and differentiation of the five groups. Furthermore, in order to reduce the time required for gel electrophoresis, the utilization of denaturing HPLC (DHPLC) for analysis of the multiplex PCR products was also investigated.

METHODS

Bacterial strains and antimicrobial susceptibility testing. Ten \textit{bla}_{CTX-M}-carrying strains were used as controls to optimize the multiplex PCR assay (Table 1). Two non-\textit{bla}_{CTX-M}-carrying \textit{Klebsiella pneumoniae} strains, WH208537 (SHV-12-producing) and WH758373 (SHV-11- and TEM-1-producing) were included as negative controls. A blinded panel of 62 \textit{bla}_{CTX-M}-positive isolates (32 \textit{Escherichia coli}, 24 \textit{Klebsiella} sp. and 6 \textit{Enterobacter cloacae}) were tested by the optimized multiplex PCR. Samples were collected between October 2003 and

Abbreviations: DHPLC, denaturing HPLC; ESBL, extended-spectrum $\beta$-lactamase.
February 2004 from 13 hospitals in the West Midlands area of the UK and between 1998 and 2001 from two hospitals in Wuhan and Beijing, China. Twenty-three of 32 Escherichia coli and 20 of 24 Klebsiella isolates were from hospital patients and the remaining 19 isolates were collected from community patients (faeces samples were submitted to Birmingham Heartlands Hospital for the diagnosis of diarrhoeal disease). The DNA sequence identity of all blaCTX-M genes of clinical isolates and control strains was confirmed by bidirectional sequencing of their PCR products using Beckman CEQ2000 protocols. The initial sample screening and antibiotic susceptibility testing were performed as described previously (Munday et al., 2004).

Multiplex PCR assay (CTX-Mplex PCR). To confirm that each primer set was sufficient for the amplification of a subgroup(s)-specific fragment, a monoplex PCR was first performed with each individual primer set using 10 phenotypically and genotypically well-characterized CTX-M-type ESBL-producing control strains. The specificity of the primers was then evaluated with the combined primer sets in the multiplex PCR with different PCR cycling parameters. PCR amplification was performed with template DNA prepared from the heat treatment of a bacterial suspension (95 °C, 5 min). Five microlitres of template DNA was added to a 50 μl reaction mixture containing 25 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP (Qiagen), all buffers at 20 to 1100 bp and all samples were analysed at 50 °C. The buffers used for the gradient were buffer A, 0.1 M triethylammonium acetate, buffer B, 0.1 M triethylammonium acetate/25% acetonitrile and buffer D, 75% acetonitrile. Peaks that exhibited an absorbance measured as greater than 1 mV were considered as genuine peaks representing PCR fragments.

RESULTS AND DISCUSSION

Identification of blaCTX-M subgroup-specific primers

The sequences of each blaCTX-M subgroup were aligned with CLUSTAL W and Genedoc. The sequence alignments were generated from 17 strains of group 1 (CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -27, -28, -29, -30, -32, -33, -34, -36, -37 and -42), 9 strains of group 2 (CTX-M-2, -4, -5, -6, -7, -20, -31, -35 and Toho-1) and 11 strains of group 9 (CTX-M-9, -13, -14, -16, -17, -18, -19, -21, -24, -38 and Toho-2). The sequences from six strains, three from group 8 (CTX-M-8, CTX-M-40 and CTX-M-41) and three from group 25/26 (CTX-M-25, -26 and -39), were aligned together. They have previously all been classified within group 8 (Tzouvelekis et al., 2000) and consequently this study will identify groups 8 and 25/26 as one group. The group-specific primer sets were designed in the highly conserved region of the alignments. The derived primer sets were subsequently aligned against all other members of different group sequences in order to reduce the risk of cross-reactivity. A total of four primer sets were identified to amplify an internal region of the blaCTX-M genes from the five groups. Primer pairs CTXM7 and CTXM8, CTXM17 and CTXM18 and CTXM11 and CTXM12 are specific to groups 1, 2 and 9, while CTXM19 and CTXM20 amplify both groups 8 and 25/26. CTXM19 and CTXM20 have 100% sequence similarity with group 25/26 and only one base mismatch in both the 5’ and 3’ ends with group 8. We were not able to test CTXM19 and CTXM20 with blaCTX-M-carrying strains as these were not available to us. The amplified multiplex PCR fragments from isolates representing groups 1, 2, 9, 8 and 25/26 ranged from 207 to 341 bp (Table 2).

### Table 1. Control strains used in evaluating CTX-Mplex PCR

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>bla genes (subgroup)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>QE15</td>
<td>Escherichia coli</td>
<td>CTX-M-15 (group 1)</td>
<td>Birmingham, UK</td>
</tr>
<tr>
<td>GZ3</td>
<td>Escherichia coli</td>
<td>CTX-M-3 (group 1)</td>
<td>Guang Zhou, China</td>
</tr>
<tr>
<td>F1</td>
<td>Escherichia coli</td>
<td>CTX-M-1 (group 1)</td>
<td>Paris, France</td>
</tr>
<tr>
<td>Y19</td>
<td>Escherichia coli</td>
<td>CTX-M-9 (group 9)</td>
<td>York, UK</td>
</tr>
<tr>
<td>Y15</td>
<td>Escherichia coli</td>
<td>CTX-M-14 (group 9)</td>
<td>York, UK</td>
</tr>
<tr>
<td>TRA</td>
<td>Escherichia coli</td>
<td>CTX-M-21 (group 9)</td>
<td>Paris, France</td>
</tr>
<tr>
<td>F2</td>
<td>Escherichia coli</td>
<td>CTX-M-2 (group 2)</td>
<td>Paris, France</td>
</tr>
<tr>
<td>TLR</td>
<td>Escherichia coli</td>
<td>CTX-M-20 (group 2)</td>
<td>Paris, France</td>
</tr>
<tr>
<td>ESBL530</td>
<td>Escherichia coli</td>
<td>CTX-M-25 (group 25/26)</td>
<td>Manitoba, Canada</td>
</tr>
<tr>
<td>H610</td>
<td>Klebsiella pneumoniae</td>
<td>CTX-M-26 (group 25/26)</td>
<td>Birmingham, UK</td>
</tr>
<tr>
<td>WH208537</td>
<td>Klebsiella pneumoniae</td>
<td>SHV-12</td>
<td>Birmingham, UK</td>
</tr>
<tr>
<td>WH758373</td>
<td>Klebsiella pneumoniae</td>
<td>SHV-11 and TEM-1</td>
<td>Birmingham, UK</td>
</tr>
</tbody>
</table>
Specificities of the multiplex PCR

The monoplex PCR amplification with 10 blaCTX-M-carrying control strains exhibited four distinct bands corresponding to the expected sizes, i.e., 207, 260, 293 and 341 bp (not shown). The specificity of the primers was then evaluated with the combined primer sets in the multiplex PCR. Using 20 pmol for each primer, a specific PCR fragment was obtained for groups 1, 2 and 25/26, and non-specific products were seen. Although the primer set for group 1 showed a lack of similarity with sequences of all the other groups, a faint cross-reactive band with group 9 was observed. To optimize the multiplex PCR assay and ensure specificity and reliability, primer sets were mixed in different ratios in multiplex PCRs. The best results, which resulted in the loss of non-specific banding from the primer pair for group 1 to group 9 template, were obtained with the following primer concentrations: 10 pmol for CTXM7 and CTXM8, 20 pmol for CTXM13, CTXM14, CTXM18 and CTXM19 and 40 pmol for CTXM11 and CTXM12. No products were obtained from control strains that produced SHV-11, SHV-12 and TEM-1 (Table 2 and Fig. 1).

Simultaneous detection of two groups of blaCTX-M

DNA templates containing combinations of CTX-M-producing control strains of groups 1, 2, 9 and 25/26 were evaluated with the multiplex PCR. The results are shown in Fig. 2(a). This experiment demonstrated that our assay could accurately detect the presence of CTX-M β-lactamase genes from two different groups within the same isolate.

The results of DHPLC analysis of multiplex PCR products are shown in Fig. 2(b). The group-specific fragments generated from multiplex PCR could be rapidly and accurately identified by non-DHPLC analysis. The various peaks at different retention times (3-13 min for group 25/26, 3-43 min for group 1, 3-64 min for group 9 and 3-90 min for group 2) correlate with the bands in Fig. 1 (single template) (group 25/26, 207 bp; group 1, 260 bp; group 9, 293 bp and group 2, 341 bp) and Fig. 2(a) (two templates) (group 1 plus group 2, 260 and 341 bp; group 25/26 plus group 9, 207 and 293 bp; group 25/26 plus group 2, 207 and 341 bp).

Evaluation of multiplex PCR with clinical isolates

The 62 clinical blaCTX-M-positive isolates included 43 from group 1 (40 CTX-M-15 and three CTX-M-3), 16 belonging to group 9 (10 CTX-M-15 and six CTX-M-9) and three CTX-M-26 producers (group 25/26). No group 2 or group 8 clinical isolates were available to us. All of the isolates were characterized in our laboratory previously by a standard PCR-based method using group-specific primers in four separate PCRs (Munday et al., 2004). Multiplex PCR testing of these 62 clinical isolates was performed in a blinded fashion. The results revealed 100% concordance with the data obtained from our standard single PCR-based assay.

Since the early 1990s, the rapid expansion of the CTX-M-type of ESBLs has created concern in the public health community worldwide. Several outbreaks caused by CTX-M-type ESBLs have been reported (Baraniak et al., 2002; Boyd et al., 2004; Woodford et al., 2004; Yan et al., 2000). In the UK, a recent study identified an epidemic CTX-M-15-producing Escheri-

Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>blaCTX-M subgroup</th>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Position (bp)</th>
<th>PCR product (bp)</th>
<th>Primer concentration (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>CTXM7</td>
<td>GCGTGATACCACTTCACCTC</td>
<td>540–559</td>
<td>260</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CTXM8</td>
<td>TGAAGTAGGTGACAGAATC</td>
<td>780–779</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Group 2</td>
<td>CTXM17</td>
<td>TGATACCACTAAGACGCAACCTC</td>
<td>543–561</td>
<td>341</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CTXM18</td>
<td>TATGTCGCTCAAGAAGCCTGGG</td>
<td>863–883</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Groups 8 and 25/26</td>
<td>CTXM19</td>
<td>CAACTGCACTTTGCGGCAATG</td>
<td>582–601</td>
<td>207</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CTXM20</td>
<td>ATACCGTGCGTGACAAATTT</td>
<td>855–873</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Group 9</td>
<td>CTXM11</td>
<td>ATCAAGGCGGCGATCTGGTATA</td>
<td>298–319</td>
<td>293</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>CTXM12</td>
<td>GTAAGCTGACGGCAACGTCTGC</td>
<td>570–590</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>
Escherichia coli with more than 100 community and in-patient isolates involved (Woodford et al., 2004). To date, in addition to 15 OXA-type, 55 SHV-type and 135 TEM-type, more than 40 CTX-M-type ESBLS have been identified (http://www.lahey.org/studies/webt.htm). To combat the technical difficulties encountered in molecular detection and characterization of these \( \beta \)-lactamases, especially ESBLS, multiplex PCR methods have been successfully developed for simultaneous amplification of three \( \beta \)-lactamase genes, \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{OXA-1}} \), and \( \text{ampC} \) (Colom et al., 2003; Perez-Perez & Hanson, 2002). The multiplex PCR assay described in this study offers an accurate, sensitive and rapid method for screening and identification of all of the \( \text{bla}_{\text{CTX-M}} \) genes encoding ESBLS. The four pairs of multiplex PCR primers designed in this study were based on sequence alignments generated from all of the published \( \text{bla}_{\text{CTX-M}} \) gene sequences in GenBank. The group-specific fragments ranging from 207 to 341 bp were clearly separated and visualized by gel electrophoresis. There was no discrepancy between results obtained by single and multiplex PCRs for all control strains evaluated. High specificity of the assay was demonstrated using 10 control strains as well as clinical isolates. During the preparation of this paper, Woodford et al. (2005) described a multiplex PCR method for the detection of CTX-M-type ESBLS; however, like the current study, they were not able to test strains from group 8 producers.

The occurrence or association of more than one \( \beta \)-lactamase within the same isolate (such as CTX-M-14 and SHV-12; CTX-M-9 and SHV-36; CTX-M-15 and TEM-2; TEM-1, OXA-1 and CTX-M-3 etc.) has been reported (Arpin et al., 2003; Chanawong et al., 2002; Colom et al., 2003; Karim et al., 2001; Munday et al., 2004), although, so far, strains carrying multiple \( \text{bla}_{\text{CTX-M}} \) genes have not been observed. However, given the nature of involvement of different genetic elements in the mobilization of \( \text{bla} \) genes, it is not impossible that dual or multiple expression of \( \text{bla}_{\text{CTX-M}} \) genes within the same isolate may arise. Thus, a method such as the multiplex PCR developed in this study that is able to detect the presence of one or more \( \text{bla}_{\text{CTX-M}} \) genes, as well as differentiate between the \( \text{bla}_{\text{CTX-M}} \) groups, can have significant clinical relevance in diagnosis, epidemiological studies and surveillance programmes.

DHPHC can be used to separate PCR products by DNA sequence and size, enabling bacterial identification and molecular characterization of antibiotic-resistance genes (Cooksey et al., 2002; Eaves et al., 2002; Hurtle et al., 2003). Accurate sizing of PCR products has been used for \( \text{Mycobacterium tuberculosis} \) typing and PCR product analysis to differentiate AmpC \( \beta \)-lactamases (Evans et al., 2004; Perez-Perez & Hanson, 2002; Tzouvelekis et al., 2000). The application of the DHPHC WAVE technology to the analysis of multiplex PCR products developed in this study has clear advantages. The PCR products are processed in a 96-well autosampler unit and injected continuously onto the column for analysis without any time-consuming pre-manipulations, such as purification and denaturation. The accuracy, flexibility and high throughput capability of multiplex-DHPLC represents a superior method for the analysis of multiplex PCR products compared with gel electrophoresis.
To conclude, we report here the development of a specific, sensitive and fast multiplex PCR assay for the detection of all bla\textsubscript{CTX-M} genes and discrimination between groups. We have also demonstrated the feasibility of utilizing DHPLC WAVE technology in screening a large number of clinical isolates at low labour cost and in a time-saving procedure.

**ACKNOWLEDGEMENTS**

We thank Dr Guillaume Arlet (Hôpital Tenon, Paris, France), Dr Michael Mulvey (National Microbiology Laboratory, Manitoba, Canada) and Dr Jianhui Xiong (representing Guangzhou Antimicrobial Resistance Surveillance Group, China) for supplying control strains.

**REFERENCES**


