A real-time multiplex PCR assay for the rapid detection of CTX-M-type extended spectrum β-lactamases directly from blood cultures

Extended-spectrum β-lactamase (ESBL) production is currently a feature of Enterobacteriaceae worldwide and CTX-M has become established as the predominant genotype (ECDC, 2010). The multiresistant nature of these organisms means that therapeutic options are limited, with carbapenems, tigecycline, colistin and fosfomycin being amongst the few agents likely to remain active in systemic sepsis.

Early antimicrobial therapy with antibiotics effective in vitro is a powerful predictor of a favourable outcome with Gram-negative sepsis (Schwaber et al., 2006). Equally, there is understandable reluctance to use these agents indiscriminately as empiric therapy.

A rapid test that can detect ESBL production in a timely fashion would improve the management of sepsis by directing both empiric therapy and infection control procedures. Currently used routine methods for the detection of ESBL-producing organisms are phenotypic and based on determining the sensitivity of an isolate to third generation cephalosporins in the presence and absence of clavulanate – this can take up to 48 h and can be unreliable (Robberts et al., 2009).

Here we describe and evaluate a relatively simple molecular method that can be performed directly on positive blood cultures that can not only detect but also differentiate between the different CTX-M-type ESBLs within 4 h.

A total of 230 blood cultures received at the Department of Medical Microbiology, the Royal Free Hampstead NHS Trust between August 2009 and August 2011 that contained Gram-negative rods were included in this study. To obtain phenotypic results, positive blood cultures were plated onto MacConkey agar (Oxoid) and incubated for 18–20 h at 35 °C. Identification and susceptibility testing were performed by using an automated analyser (Phoenix, BD Diagnostics) following the manufacturer’s instructions. Isolates showing resistance to the indicator cephalosporins (ceftazidime and cefepoxide) by Phoenix methodology were tested by combination disc diffusion tests using cefpodoxime and cepfepime with and without clavulanate, following Health Protection Agency (HPA) guidelines (Livermore & Woodford, 2004). Isolates with ≥5 mm difference in zone diameter between the cephalosporin discs with and without clavulanate after 20 h incubation were interpreted as ESBL producers.

To obtain PCR results, bacterial DNA was extracted directly from 3 μl positive blood cultures by using a BD GeneOhm Lysis kit (BD Diagnostics), following the dilution method as per the manufacturer’s instructions. The pan-CTX-M PCR was performed in a final volume of 20 μl on a RotorGene3000 apparatus (Qiagen). PCR conditions were 10 min incubation period at 95 °C, followed by 35 cycles of 8 s at 95 °C, followed by 60 s at 58 °C, with a single fluorescence reading for all four channels being taken at the end of the extension stage [previously described by Birkett et al. (2007)], using primers and probes as listed in Table 1.

DNA extracted from control isolates producing CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group and CTX-M-25 group ESBLs (NCTC 13461, NCTC 13462, NCTC 13463, NCTC 13464 and NCTC 13465, respectively, provided by the Antibiotic Resistance Monitoring Reference Laboratory, HPA, London) was included in every PCR assay. To determine the detection limit of the assay, these control strains were spiked into negative blood cultures to give concentrations of between 1 × 10^6 and 1 × 10^9 c.f.u. ml⁻¹ – the DNA extraction and PCR results showed a detection limit of approximately 1 × 10^6 c.f.u. ml⁻¹ for all CTX-M groups. For comparison, the number of bacteria in ten Gram-negative rod-containing clinical blood culture broths when they flagged positive on an automated system (Bactec FXTM, BD Diagnostics) was determined. The positive cultures contained an average of 6.5 × 10^8 c.f.u. ml⁻¹ – more than 60 000 times higher than the limit of detection of the PCR assay.

Phenotypic testing showed that 12/230 (5.2 %) of the blood cultures contained an ESBL-producing organism – nine isolates were identified by the Phoenix as Escherichia coli and three as Klebsiella pneumoniae. The real-time multiplex PCR correctly detected 11 of the 12 ESBL-producing organisms directly from the blood culture broths and genotyped them into the CTX-M-1 group (which contains CTX-M-1, -3, -10, -11, -12, -15, -28 and -30). The one isolate in which ESBL production was detected by phenotypic testing but not by the pan-CTX-M PCR was identified as K. pneumoniae. The ESBL genotype was shown to be OXA-30, using a conventional PCR method described by Dallenne et al. (2010), which explains this discrepancy. The real-time PCR (RT-PCR) did not give any false-positive results.

To confirm that the CTX-M-1 group ESBL genes had been detected by the RT-PCR assay, products from six of the positive blood cultures were purified using the QIAquick PCR purification kit (Qiagen) and sequenced in both directions using the pan-CTX-M primers (Table 1) on an ABI3130 Genetic Analyser (Applied Biosystems). Sequences were edited using BioNumerics version 6.5 and nucleotide BLAST (GenBankNational Center for Biotechnology Information) analysis of the resulting amplicons confirmed that they were all CTX-M-1 group ESBLs; four gave a 100 % match with blacTX-M-1 and two gave a 100 % match with blacTX-M-15.

Results indicating the presence of ESBL genes were available by RT-PCR within 3.5 h of the initial Gram stain – more than
offset by the cost saving observed across £5 per PCR assay) would be more than increased cost to the laboratory (approx. unavoidable in clinical laboratories. The An element of batching will, however, be of particular benefit in areas and patient blood cultures that have flagged positive. should be performed as soon as possible maximum effect, DNA extraction and PCR an ESBL-producing organism. For adjunct to routine phenotypic techniques producers is high and should be used as an advantage of identifying the CTX-M genotype, which will inform infection control investigations. In addition, results from this assay are not affected by many of the problems inherent with phenotypic testing, such as substrate preference and inoculum variability. As this technique detects a specific CTX-M target, it could be adapted for use on other samples including those that might contain polymicrobial microbiota.

This simple and rapid technique has the potential to dramatically improve patient outcome and facilitate both antibiotic stewardship and infection control practices much faster than currently possible. It will be of particular benefit in areas and patient populations where the prevalence of ESBL producers is high and should be used as an adjunct to routine phenotypic techniques to provide an early alert of the presence of an ESBL-producing organism. For maximum effect, DNA extraction and PCR should be performed as soon as possible after Gram-negative rods are detected in blood cultures that have flagged positive. An element of batching will, however, be unavoidable in clinical laboratories. The increased cost to the laboratory (approx. £5 per PCR assay) would be more than offset by the cost saving observed across the health care setting as a result of decreased lengths of stay for patients owing to earlier initiation of appropriate antibiotic therapy. Further cost savings could be expected from the lower rates of health-care-associated infection that would result from more effective infection control.

Further evaluation of this assay, along with the development of real-time multiplex PCR assays for the detection of non-CTX-M ESBL- and AmpC cephalosporinase-producing organisms, is currently under way.

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Table 1. Sequences of the primers and probes used [adapted from Birkett et al. (2007) following advice from the authors]

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence (5′–3′)*</th>
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<tbody>
<tr>
<td>Forward CTX-M consensus primer</td>
<td>ATGTGCAGYACCTAGTAARGTAKGAC</td>
</tr>
<tr>
<td>Reverse CTX-M consensus primer</td>
<td>ATCACKCGGRTCGCCXGGRAT</td>
</tr>
<tr>
<td>CTX-M-1 group probe</td>
<td>Joe-CGGACACGCTGGGAGAAGGT-BHQ1</td>
</tr>
<tr>
<td>CTX-M-2 group probe</td>
<td>Cy5-CAGGTGCTTATGGCTGCTCTGTGTT-BHQ-3</td>
</tr>
<tr>
<td>CTX-M-9 group probe</td>
<td>Rox-CTGGATCGCAGCAGCTACGCTGA-BHQ-2</td>
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<tr>
<td>CTX-M probe for all groups except CTX-M-1</td>
<td>6-Fam-CGACAATAACNGCATGAA-MGB-NFQ</td>
</tr>
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*N, A or T or G or C; K, G or T; R, A or G; Y, C or T; X, inosine.