MINIREVIEW

Detection of $\text{bla}_{\text{SHV}}$, $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{CTX-M}}$ antibiotic resistance genes in randomly selected bacterial pathogens from the Steve Biko Academic Hospital

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Abstract

Extended-spectrum β-lactamases (ESBLs) are considered to be one of the most important antibiotic resistance mechanisms. This study reported the ESBL-producing genes in 53 randomly selected clinical bacterial isolates from the Steve Biko Academic Hospital. The presence of the $\text{bla}_{\text{SHV}}$, $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{CTX-M}}$ genes was determined, and the overall prevalence of these genes detected in this study was 87% (46/53) in comparison with the literature; these results were higher when compared with 33% for *Escherichia coli* in Europe and 0.8% in Denmark for similar pathogens. These research findings indicated that it is crucial to routinely monitor the prevalence of these resistance genes.

Introduction

Extended-spectrum β-lactamases (ESBLs) were first identified in the early 1980s in Germany and have since been identified worldwide (Winkokur *et al*., 2001; Meyer *et al*., 2004). The ESBL genes have been found in a number of different bacteria, including *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis* and *Salmonella* species (Paterson, 2000; Winkokur *et al*., 2001). β-Lactamase-producing bacteria are increasingly being reported as the cause of severe infections in intensive care and surgical units (Samaha-Kfoury & Araj, 2003; Solorzano *et al*., 2004). Infections associated with ESBL-producing bacteria include central venous line-related bacteraemia, cholangitis, intra-abdominal abscesses, peritonitis and urinary tract infections (Paterson, 2000). In hospitalized patients, ESBL producers may cause nosocomial pneumonia and meningitis (Paterson, 2000). Mortality rates varying from 42% to 100% have been reported in patients infected with ESBL-producing bacteria (Colodner, 2005).

ESBL-producing bacteria often show cross-resistance with other groups of antibiotics, such as fluoroquinolones (Colodner, 2005). It was indicated that 18% of ESBL-producing isolates in the United States were also ciprofloxacin resistant (Colodner, 2005). The early detection of ESBLs is therefore extremely important, because the prevalence of ESBLs is severely underestimated (Colodner, 2005).

Most ESBL-producing bacteria can be divided into three groups: TEM, SHV and CTX-M types (Pitout & Laupland, 2008). Gram-negative β-lactamases are often mediated by $\text{bla}_{\text{SHV}}$, $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{CTX-M}}$ genes (Tzouvelekis *et al*., 2000; Monstein *et al*., 2007). The SHV enzymes are named after the sulphydryl variable active site and are commonly associated with *K. pneumoniae* (Samaha-Kfoury & Araj, 2003). Initially, these bacteria contained a single ESBL gene, but now multiple ESBL genes are commonly present in a single strain, further complicating the process of detection (Samaha-Kfoury & Araj, 2003). The TEM-type ESBL are derivatives of TEM-1 and TEM-2, and currently, TEM-3 has been discovered, differing from TEM-2 by two amino acid
substitutions (Al-Jasser, 2006). The original TEM was first discovered in *E. coli* in Greece, but it spread rapidly to other genera of the *Enterobacteriaceae* (Samaha-Kfouri & Araj, 2003) such as *Citrobacter freundii* and *Acinetobacter* spp. (Samaha-Kfouri & Araj, 2003; Colodner, 2005). More recently, *Enterobacteriaceae* producing novel ESBLs such as CTX-M enzymes have emerged within hospitals and communities worldwide (Pitout et al., 2006). The name CTX is an abbreviation for cefotaximase and refers to the potent hydrolytic activity of this enzyme against cefotaxime (Tzouvelekis et al., 2000). The CTX-M β-lactamases exceed > 50 different types based on their amino acid identities and can be divided into five groups: the CTX-M group 1 (including CTX-M-1, -3, -10, -12, -15, -28 and -30, and FEC-1); the CTX-M group 2 (including CTX-M-2, -4, -5, -6, -7 and -20, and Toho-1); the CTX-M group 8 (including CTX-M-8); the CTX-M group 9 (including CTX-M-9, -13, -14, -16, -17, -19, -21, -24 and -27, and Toho-2); and the CTX-M group 25 (including CTX-M-25 and -26) (Perez et al., 2007).

The choice of drugs for the treatment of ESBL-producing bacteria is limited to carbapenems, for example imipenem, or, alternatively, fluoroquinolones and aminoglycosides, which may be used if these antibiotics show in vitro activity (Samaha-Kfouri & Araj, 2003). Third-generation cephalosporins should not be used to treat serious infections with ESBL-producing bacteria, making the carbapenems, including imipenem and etrapenem, the widely recognized first-choice drug (Pitout & Laupland, 2008). A study during January 2001 to December 2004 in Spain showed that 73.4% (113/154) of clinical isolates produced ESBLs (Romero et al., 2007), while a prevalence of 63% (832/1 313) was detected in 2007 (Bell et al., 2007), and a final extension step at 72°C of 1 1000 (Qiagen, Hilden, Germany) for the simultaneous detection of the SHV (747 bp), CTX-M (593 bp) and TEM (445 bp) genes. Three sets of primers specific for CTX-M, SHV and TEM genes were used, as shown in Table 1. All the primers were manufactured by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). The M-PCR assay was performed using the Qiagen Multiplex PCR Kit 1000 (Qiagen, Hilden, Germany) for the simultaneous detection of the SHV (747 bp), CTX-M (593 bp) and TEM (445 bp) genes. Three sets of primers specific for CTX-M, SHV and TEM genes were used, as shown in Table 1. The 25-µL final reaction mixture consisted of 12.5 µL, 2 x Qiagen Multiplex PCR Mastermix (HotStart Taq DNA polymerase, Multiplex PCR buffer and dNTP Mix), Q-solution 5 x and nuclease-free water (Qiagen) and a volume of 4 µL of the prepared DNA template. The reaction tubes were placed in a PX2 Thermal cycler (Thermo Electron Corporation, MA distributed by the Scientific Group, SA). The cycling conditions were as follows: denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. A previously identified *K. pneumoniae* ESBL-positive isolate was used as a positive control, and a negative control (nuclease-free water) was included in each run (Meyer et al., 2007).

### Materials and methods

**Sample analysis**

Fifty-three randomly selected ESBL-producing clinical bacterial isolates were obtained, over a 2-week period in August 2007, from clinical specimens sent from an academic hospital for analysis to the diagnostic laboratory at the Department of Medical Microbiology, Faculty of Health Science, University of Pretoria/NHLS, South Africa. The ESBL-producing genes were detected in the following isolates: one *C. freundii*, 13 *E. coli*, five *Morganella morganii* spp. *morganii*, two *Enterobacter cloacae*, 31 *K. pneumoniae* and one *Proteus penneri*.

### Antibiotic resistance determination and bacterial DNA extraction

Identification and antibiotic resistance was determined using the Vitek 2 System (bioMérieux, France) according to the manufacturer’s protocol. The MagnaPure LC Compact (Roche Applied Science, Germany) was used for the automated extraction of total DNA (according to the manufacturer’s protocol). Bacterial cultures were inoculated in 5 mL of brain heart infusion broth (Merck, South Africa) and incubated (Labcon, South Africa) for 24 h at 37°C. A volume of 400 µL of the 24-h incubated cultures was used for automated DNA extraction. DNA was eluted in a final volume of 100 µL of elution buffer (Roche Applied Science). The extracted DNA was stored at −70°C for further analysis.

### M-PCR amplification

The M-PCR amplification was performed according to the method of Monstein et al. (2007). The primer-pair sequences used in the M-PCR assay, melting temperatures, expected PCR and amplicon sizes are given in Table 1. All the primers were manufactured by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). The M-PCR assay was performed using the Qiagen Multiplex PCR Kit 1000 (Qiagen, Hilden, Germany) for the simultaneous detection of the SHV (747 bp), CTX-M (593 bp) and TEM (445 bp) genes. Three sets of primers specific for CTX-M, SHV and TEM genes were used, as shown in Table 1. The 25-µL final reaction mixture consisted of 12.5 µL, 2 x Qiagen Multiplex PCR Mastermix (HotStart Taq DNA polymerase, Multiplex PCR buffer and dNTP Mix), Q-solution 5 x and nuclease-free water (Qiagen) and a volume of 4 µL of the prepared DNA template. The reaction tubes were placed in a PX2 Thermal cycler (Thermo Electron Corporation, MA distributed by the Scientific Group, SA). The cycling conditions were as follows: denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. A previously identified *K. pneumoniae* ESBL-positive isolate was used as a positive control, and a negative control (nuclease-free water) was included in each run (Meyer et al., 2007).
Single PCR amplification and sequencing of the CTX-M group

The CTX-M-positive isolates identified by M-PCR were further analysed using group-specific primer-pair sequences to distinguish the specific CTX-M groups [Inqaba Biotechnical Industries (Pty) Ltd]. The CTX-MA1 and CTX-MA2 primers, according to the method of Pitout et al. (2007), were used to detect CTX-M group 1, CTX-M group 2, CTX-M group 8 and CTX-M group 9 (Table 1). The 25-μL PCR reaction mixture consisted of 12.5 μL of Master Mix HotStart Taq (Qiagen), 3 μL nuclease-free water (Qiagen), 2.5 μL Q-solution (Qiagen), 1.5 μL of each of the primers and a volume of 4 μL of the prepared DNA template. The reaction tubes were placed in a PX2 Thermal cycler (Thermo Electron Corporation). The cycling conditions were as follows: denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min.

Detection of amplification products

The amplicons (products) of the PCR reactions were visualized using an UltraViolet light box (UVP products), following electrophoresis on a 2% (m/v) agarose (Whitehead Scientific, Cape Town) gel, which contained 11.5 μL of ethidium bromide (0.5 mg mL⁻¹) (Promega, Madison). A molecular weight (50-bp ladder) marker (Promega) was used as a reference.

Sequencing of selected CTX-M-positive isolates

Confirmation and further identification was completed after the PCR assay and DNA sequence analysis were performed. Eight of the CTX-M1-positive isolates were randomly selected and sent to Inqaba Biotechnical Industries (Pty) Ltd for sequencing. The sequencing results were analysed using the BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST 2.2.18) (Bell et al., 2007).

Results and discussions

In this study, 53 clinical isolates obtained for the Steve Biko Academic Hospital were analysed using an M-PCR assay for the simultaneous detection of the SHV, CTX-M and TEM genes. Positive CTX-M isolates were further analysed using a single PCR assay to determine the specific groups.

The overall prevalence of these ESBL genes in this study was 87% (46/53). M-PCR simultaneously amplified and detected the presence of \( \text{bla}_{\text{SHV}} \) (747 bp), \( \text{bla}_{\text{CTX-M}} \) (593 bp) and \( \text{bla}_{\text{TEM}} \) (445 bp), respectively (Fig. 1). The M-PCR assay identified the results for each of the resistance genes as follows: the \( \text{bla}_{\text{TEM}} \) gene only, detected in 24% (13/53); the \( \text{bla}_{\text{SHV}} \) gene in 4% (2/53); \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) detected in 12% (6/53); \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{CTX-M}} \) detected in 12% (6/53); and \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{CTX-M}} \) detected in 36% (19/53) of the isolates. The last 12% (6/53) isolates were negative for any of the resistance genes and could be false-positive Vitek results. The results of an M-PCR assay study, conducted between 2001 and 2003 in Sweden by Monstein et al. (2007), yielded the following conclusions: \( \text{K. pneumoniae} \): \( \text{bla}_{\text{SHV}} \) detected in 8% (3/37), \( \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{TEM}} \) detected in 2.7% (1/37), and \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{CTX-M}} \) detected in 8% (3/37) isolates (Monstein et al., 2007). The ESBL prevalence in the study conducted at the Steve Biko Academic Hospital showed a higher prevalence compared with the Sweden results. The variation in the prevalence of ESBL-producing isolates in the Steve Biko Academic Hospital could be a result of poor infection control, spreading of hospital-acquired ESBLs into the community and vice versa.

The single PCR amplification assay results to detect the different CTX-M groups showed that 47% (25/53) isolates were CTX-M group 1 positive, while no isolates were positive for any of the other CTX-M groups. Because the current concern is mainly based on the detection of...
CTX-M-15, which belongs to the CTX-M group 1, only eight CTX-M group 1-positive isolates were randomly selected and sequenced. The sequencing results of the eight randomly selected isolates were 100% (8/8) positive for CTX-M-15, confirming circulation of those strains in various wards associated with different infections in the Steve Biko Academic Hospital, Pretoria, South Africa. A study by Eisner et al. (2006), conducted between 1998 and 2004 in Austria, showed that 78% (38/49) of the CTX-M producers carried group 1 enzymes (Eisner et al., 2006). Previous studies by Naseer et al. (2006) also reported a nosocomial outbreak of CTX-M-15 in Scandinavia, mainly in urinary tract isolates (Naseer et al., 2006). However, the CTX-M-15 enzyme in the Steve Biko Academic Hospital were detected in patients with urinary tract infections 12.5% (1/8), wound sepsis 50% (4/8) and head injuries 37.5% (3/8). Resistance because of CTX-M-15 was detected throughout the hospital in wards such as cardiothoracs, trauma ICU, urology, antenatal as well as neurosurgery. In recent studies by Pitout et al. (2005), CTX-M-15 caused both hospital- as well as community-based outbreaks, which is worrisome, because it only occurred in hospital settings previously (Pitout et al., 2005). The CTX-M-15 enzyme is increasingly seen in multidrug-resistant strains showing resistance to at least two of the following antibiotics: fluoroquinolones, tetracycline and aminoglycosides (Pitout et al., 2005). These findings emphasize the importance of rapid and accurate detection of these resistance genes to ensure appropriate infection control and the importance of continued surveillance programmes in hospital settings (Kim et al., 2007).

The current concern, however, is the increase in the prevalence of CTX-M groups in the hospital setting as well as the community (Pitout et al., 2005). Escherichia coli producing CTX-M-15 has become widely distributed throughout the United Kingdom, as indicated in a study conducted during 2003 (Pitout et al., 2007). A study conducted by Rodríguez-Baño et al. (2006) showed that patients infected with the CTX-M-14-producing isolates had acquired the organism in the community (Rodríguez-Baño et al., 2006).

**Conclusion**

ESBLs are considered to be one of the most important antibiotic resistance mechanisms (Samaha-Kfoury & Araj, 2003). Multidrug resistance is emerging in many Gram-negative pathogens and is associated with severe nosocomial infections (Pitout & Laupland, 2008). In order to eradicate the spread and transmission of these ESBL-producing strains, rapid diagnostic techniques such as M-PCR, to detect these strains, have to be implemented for the successful surveillance and for the implementation of the correct treatment of these strains in hospitals (Pitout & Laupland, 2008).

The occurrence of CTX-M enzymes presents problems in the hospital setting, complicating treatment for diseases such as urinary tract infections (Woodford et al., 2004). The occurrence of CTX-M enzymes has an effect on public health such as spreading of hospital-acquired infections to the community (Woodford et al., 2004). The detection of these CTX-M-resistant genes in the Steve Biko Academic Hospital, which is in agreement with similar findings worldwide, emphasizes the epidemic potential of multiple-antibiotic-resistant CTX-M in countries (Naseer et al., 2006).
These findings were supported by the fact that many CTX-M cases occurred early during hospitalization (Rodríguez-Baño et al., 2006). These significant public health implications mean that the spread of bacteria producing ESBLs (particularly CTX-M enzymes) requires close monitoring, with enhanced surveillance (Lewis et al., 2007), and changes have to be made in the antibiotic utilization policies, with careful consideration, before the resistance predicament worsens (Stürenburg & Mack, 2003).

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References

