A simple disc diffusion method for detecting AmpC and extended-spectrum β-lactamases in clinical isolates of Enterobacteriaceae

Helen Derbyshire¹, Gemma Kay², Katie Evans³, Carmel Vaughan³, Umadevi Kavuri⁴ and Trevor Winstanley¹*

¹Department of Microbiology, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF, UK; ²Biomedical Research Centre, Faculty of Health and Wellbeing, Sheffield Hallam University, Sheffield S1 1WB, UK; ³Mast Diagnostics, Mast House, Derby Road, Bootle L20 1EA, UK; ⁴School of Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK

Received 27 October 2008; returned 11 December 2008; revised 15 December 2008; accepted 15 December 2008

Background: We sought to determine whether extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases (derepressed and inducible), alone and in combination, could be detected in unidentified members of the Enterobacteriaceae using a simple, overnight disc diffusion test.

Methods: The genetic basis of antibiotic resistance in cephalosporin-resistant wild-type (n=140) and culture collection (n=140) isolates of Enterobacteriaceae was determined using PCR. A scheme for detecting these resistance mechanisms phenotypically was devised using five antibiotic discs: cefpodoxime + clavulanate; cefepime + clavulanate and cefoxitin.

Results and conclusions: AmpC β-lactamases (derepressed and inducible) and ESBLs, alone and in combination, could reliably be detected using a disc diffusion method. ESBLs alone could be detected on the basis of a difference of >5 mm between cefpodoxime/clavulanate and cefpodoxime (10 µg) discs. ESBLs, in the presence of AmpC β-lactamases, could be detected using a difference of >5 mm between cefepime/clavulanate and cefepime (30 µg) discs. AmpC β-lactamases could be detected using a difference of >14 mm between cefepime/clavulanate and cefpodoxime/clavulanate discs. Inducible AmpC β-lactamases could be discerned by observing the blunting of the cefpodoxime or cefpodoxime/clavulanate zones in proximity to cefoxitin (30 µg) discs.

Keywords: ESBLs, resistance phenotypes, mechanisms of resistance

Introduction

Members of the Enterobacteriaceae commonly express plasmid-encoded β-lactamases (e.g. TEM-1, TEM-2, SHV-1) that confer resistance to penicillins, but not to cephalosporins with oxyimino side chains.

Resistance to these extended-spectrum cephalosporins appeared initially in a limited number of genera (Enterobacter cloacae, Citrobacter freundii, Serratia marcescens and Morganella morganii) that could hyper-produce AmpC β-lactamases. Such β-lactamases are typically encoded on the chromosome where their expression may be inducible. When induced or derepressed, they hydrolyse cephapemycins (cefoxitin and cefotetan) as well as third-generation cephalosporins (cefotaxime, ceftiraxone and cefazidime) and monobactams (aztreonam) but are poorly inhibited by β-lactamase inhibitors such as clavulanic acid.¹

In the mid-1980s,² extended-spectrum β-lactamases (ESBLs) emerged. These evolved via point mutations of TEM-1, TEM-2 and SHV-1 genes and were capable of hydrolysing third-generation cephalosporins and monobactams. In contrast to AmpC β-lactamases, they are not active against cephamycins but are susceptible to β-lactamase inhibitors (e.g. clavulanic acid).¹ These mutant enzymes were most often found in nosocomial Klebsiella spp. from patients in intensive care or other specialist settings.

From 2000 onwards, however, CTX-M ESBLs began to predominate, mainly in Escherichia coli and predominantly at the hospital/community interface. These enzymes generally have greater activity against cefotaxime than other oxyimino-β-lactam
Methods and materials

Consecutive, non-replicate isolates of Enterobacteriaceae that were resistant to cefpodoxime (urinary isolates, \(n = 74\)) or cefazidime and/or cefotaxime (systemic isolates, \(n = 66\)) were collected from The Royal Hallamshire Hospital, Sheffield (January 2008–March 2008). They were identified by standard agar incorporation methods and comprised \(E. coli\) (39 urinary, 13 systemic), \(Klebsiella\) pneumoniae (33), \(K. oxytoca\) (5), \(P. mirabilis\) (4), \(E. agglomerans\) (2); \(E. cloacae\) (23), \(E. coli\) (18), \(C. freundii\) (16), \(E. aerogenes\) (16), \(E. aerogenes\) (9), \(M. morganii\) (8), \(S. marcescens\) (3/2), \(Hafnia alvei\) (0/1) and \(Providencia alcalifaciens\) (0/1). AmpC β-lactamase producing genes (\(MOX-1, MOX-2, CMY-1, CMY-8\) to \(CMY-11\); \(LAT-1\) to \(LAT-4\); \(CMY-2\) to \(CMY-7\); \(BIL-1\); \(DHA-1, DHA-2\); \(ACC\); \(MI-1, ACT-1\); \(FOX-1\) to \(FOX-5b\)) were sought in the 140 clinical isolates by PCR using the method of Pérez-Pérez and Hanson with the exception that 4.5 mL of overnight culture was used instead of 1.5 mL. SHV- and TEM-derived ESBL genes were sought using the PCR methodology of Naiemi et al. and CTX-M genes were sought by the method of Bouallégue-Goet et al. Resistances due to K1 \(E. coli\), hyper-production of β-lactamase and impermeability were discerned by interpretative reading.

Genotypically characterized culture collection strains (\(n = 106\)) were also included in the study as were 34 characterized isolates kindly supplied by Dr Mandy Wootton, University Hospital of Wales. Together, these comprised \(E. coli\) (55), \(K. pneumoniae\) (35), \(E. cloacae\) (17), \(K. oxytoca\) (14), \(E. aerogenes\) (7), \(Proteus mirabilis\) (5), \(E. agglomerans\) (3), \(C. freundii\) (3) and \(M. morganii\) (1). Isolates were processed in a blind fashion with PCR for ESBL genes, PCR for AmpC genes and disc diffusion carried out at different centres. Disc diffusion tests were carried out according to BSAC methodology, using paired discs prepared to FDA limits. Briefly, cefpodoxime discs (10 μg) + clavulanate (Mast Diagnostics, Derby Road, Boatle, UK) and cefepime discs (30 μg) + clavulanate were applied using a standard disc dispenser. A cefoxitin disc (30 μg) was then applied by forceps so that it was 15 mm (centre-to-centre) from the cefpodoxime and cefpodoxime/clavulanate discs that were adjacent to each other. After overnight incubation, zone diameters were read using a MastascanElite automated reader (Mast Diagnostics). Zone diameters were entered into Microsoft Excel and the difference in zone diameters between cefpodoxime/clavulanate and cefepime, cefepime/clavulanate and cefepime/clavulanate and cefpodoxime/clavulanate was calculated. Blunting of the cefpodoxime + clavulanate zones by cefoxitin was also noted.

Results and discussion

Of the 280 isolates tested (140 clinical and 140 reference), PCR revealed the following genes: ESBL [\(n = 117\): \(E. coli\) (73), \(K. pneumoniae\) (33), \(K. oxytoca\) (5), \(P. mirabilis\) (4), \(E. agglomerans\) (2)]; AmpC [\(n = 99\): \(E. cloacae\) (23), \(E. coli\) (18), \(C. freundii\) (16), \(E. aerogenes\) (16), \(E. aerogenes\) (9), \(M. morganii\) (8), \(S. marcescens\) (3/2), \(H. alvei\) (0/1)] and \(P. alcalifaciens\) (0/1); ESBL + AmpC [\(n = 32\): \(E. cloacae\) (11), \(E. agglomerans\) (7), \(E. aerogenes\) (4), \(E. coli\) (4), \(C. freundii\) (3), \(K. pneumoniae\) (2), \(P. mirabilis\) (1); neither \(n = 31\)]. Phenotypically, AmpC genes were inducible on 30 occasions (5 in the presence of an ESBL), and the majority of these (24) were found in clinical isolates. All six AmpC pools were detected overall. Of the 31 isolates not displaying ESBL or AmpC genes, 5 were thought to be hyper-producers of β-lactamase (K. pneumoniae), 12 were impermeable (\(E. coli\)) and 14 harboured K1 β-lactamase (\(K. oxytoca\)).

All 117 ESBL-producing strains (together with 5 in the presence of an inducible AmpC) gave a cefpodoxime/clavulanate zone diameter of >5 mm greater than the corresponding cefpodoxime zone diameter (Figure 1a). Isolates producing an AmpC β-lactamase (derepressed or inducible) and those producing neither an AmpC β-lactamase nor an ESBL produced a zone difference of ≤5 mm as did those producing both an ESBL and an AmpC β-lactamase. This highlights the deficiency in using cefpodoxime + clavulanate for the detection of ESBLs in the presence of AmpC β-lactamases.

Using a similar strategy with cefepime/clavulanate and cefepime discs, 25 ESBL-producing isolates would have been missed and this emphasizes the fact that these discs cannot be recommended as a single screen for ESBLs in Enterobacteriaceae. However, their strength lies in the fact that the combination can detect ESBLs in the presence of AmpC β-lactamases (all 32 isolates detected; Figure 1b). In a survey of 32 027 consecutive clinical isolates of Gram-negative bacteria, we showed that 24%
Figure 1. Distribution of difference in zone diameters between (a) cefpodoxime/clavulanate and cefpodoxime, (b) cefepime/clavulanate and cefepime and (c) cefepime/clavulanate and cefpodoxime/clavulanate discs.
of ESBL producers co-produced an AmpC β-lactamase (data not shown). Isolates producing an AmpC β-lactamase alone (derepressed or inducible) and those producing neither an AmpC β-lactamase nor an ESBL produced a zone difference of ≤5 mm between cefepime/clavulanate and cefepime discs.

A breakpoint of >14 mm between cefepime/clavulanate and cefpodoxime/clavulanate zone diameters differentiated AmpC β-lactamase producers (alone or in the presence of an ESBL) from the remaining organisms (Figure 1c). The presence of an inducible AmpC β-lactamase was discerned by observing the blunting of the cefpodoxime or cefpodoxime/clavulanate zone by cefoxitin. Extended-spectrum AmpC β-lactamases, characterized by increased catalytic activity against oxyimino-cephalosporins including cefepime, were not detected in this study. Nonetheless, isolates resistant to cefepime would merit further study.

The strategy for examination of isolates is thus: screen with cefpodoxime or with cefotaxime and/or ceftazidime and then test any resistant isolates with the five-disc combination. The method may also be used to provisionally detect resistant organisms in blood cultures yielding Gram-negative bacteria or as a follow-up for isolates initially tested by automated methods. The use of five discs in this configuration permits two further discs to be added to the plate and these may be antibiotics used for specific treatment of resistant organisms, e.g. carbapenems (meropenem or ertapenem), temocillin or fosfomycin.

There is clearly a need for a simple test to discern ESBL-producing organisms and AmpC-β-lactamase-producing organisms. For example, UKNEQAS distribution 2355 (25 August 2008) was an E. coli that produced a plasmid-mediated AmpC β-lactamase; however, 24% of participants incorrectly reported that the organism was an ESBL producer (Christine Walton, personal communication).

Acknowledgements

We are grateful to Dr Mandy Wootton and Dr Robin Howe (University Hospital of Wales) for supplying isolates and to the Biomedical Research Centre (Sheffield Hallam University) for laboratory facilities.

Funding

No external funding was provided for this work. Mast Diagnostics provided consumables to G. K. for the detection of ESBL genes.

Transparency declarations

T. W. has received speaking invitations from various pharmaceutical companies and has provided consultancy for various diagnostic companies but these do not pose a conflict of interest with this work. Other authors: none to declare.

References

Detection of AmpC and extended-spectrum β-lactamases


