Comparison of methods for AmpC β -lactamase detection in *Enterobacteriaceae*

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AmpC β -lactamases (Bla_{AmpC}) are an emerging group of antimicrobial resistance determinants. The lack of an agreed Bla_{AmpC} detection method hinders investigation of their epidemiology and understanding of their clinical significance. This study compared the sensitivity and specificity of phenotypic methods of Bla_{AmpC} detection in a collection of 246 *Enterobacteriaceae* with a diverse range of β -lactam resistance profiles. The Bla_{AmpC} screening methods evaluated were based on cephamycin, ceftazidime and cefepime susceptibility. These were compared with Bla_{AmpC} screening using conventional ESBL detection methods. The confirmatory methods evaluated were biologically based assays, inhibitor-based assays, an AmpC Etest and a rapid chromogenic assay. A multiplex nucleic acid amplification test and the three-dimensional enzyme extraction assay were used as reference methods. Bla_{AmpC} activity was present in 74 isolates. The majority of the enzymes were plasmid-encoded and belonged to the CMY, DHA and EBC families. The screening methods had sensitivities between 47 and 99 % and specificities of 45–95 %. The performance of confirmatory tests varied widely, ranging in sensitivity from 19 % to 97 % and in specificity from 88 % to 100 %. Only the Tris-EDTA and MAST ID D68C disc tests had a sensitivity and a specificity above 90 %. Further investigation is needed to establish the most suitable enzyme substrates, inhibitor types, inhibitor concentrations and interpretative cut-offs in order to refine the inhibitor-based methods. A simple disc-based protocol using cefoxitin non-susceptibility as a screening tool, followed by the Tris-EDTA method for confirmation, detects Bla_{AmpC} activity with 95 % sensitivity and 98 % specificity.

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INTRODUCTION

In spite of the recent decision by both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to lower expanded-spectrum cephalosporin breakpoints for *Enterobacteriaceae*, there remains a need to detect and characterize specific resistance determinants such as extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases (Bla_{AmpC}) for epidemiological surveillance and infection control purposes (Doi & Paterson, 2007). Although less prevalent than ESBLs (Jacoby, 2009), Bla_{AmpC} have a comparatively broader substrate range, are not inhibited by traditional β -lactamase inhibitors and bacteria producing them are more likely to become resistant to carbapenems (Philippon *et al.*, 2002). Whereas standardized screening and confirmatory methods for ESBL identification are agreed upon (CLSI, 2010), no such methods for Bla_{AmpC} detection exist.

Members of the family *Enterobacteriaceae* such as *Enterobacter* spp., *Citrobacter freundii* and *Serratia marcescens* possess chromosomally encoded Bla_{AmpC}, obviating the need for specific Bla_{AmpC} testing for these species (Thomson, 2010). Chromosomally encoded Bla_{AmpC} in *Escherichia coli* is accompanied by negligible expression because of the presence of a transcriptional attenuator coupled with a weak promoter (Tan *et al.*, 2009). Infrequently, hyperproduction of Bla_{AmpC} in *E. coli* arises following mutations in these regulatory regions or acquisition of imported Bla_{AmpC} genes (Mulvey *et al.*, 2005). Most of the remaining clinically important *Enterobacteriaceae*, including *Klebsiella* spp., *Proteus mirabilis* and *Salmonella* spp., lack chromosomally encoded Bla_{AmpC} but the emergence of plasmid-encoded Bla_{AmpC} in the last few decades has altered the situation. The mobility afforded by transferable genetic elements has enabled rapid global dissemination reminiscent of the emergence of ESBLs (Kohner *et al.*, 2009). For example, in China the prevalence of *E. coli* and *Klebsiella pneumoniae* expressing plasmid-encoded Bla_{AmpC} increased from 2 % to 9 % between 2005 and 2006 (Ding *et al.*, 2008).

Abbreviations: Bla_{AmpC}, AmpC β -lactamase(s); ESBL, extended-spectrum β -lactamase.

A number of detection methods for *Bla*_{AmpC} have been proposed. Potential screening tools include reduced susceptibility to cephamycins and/or ceftazidime (Moland *et al.*, 2002), cefepime susceptibility (Doi & Paterson, 2007) and a positive ESBL screening test (Munier *et al.*, 2010) with or without a negative confirmatory test (Doi & Paterson, 2007), or combinations of these. More specific, but more time- and labour-intensive tests may be used to confirm the presence of *Bla*_{AmpC}, including biological assays [e.g. the modified cefoxitin Hodge test (Yong *et al.*, 2002) and Tris-EDTA disc test (Black *et al.*, 2005a)], inhibitor-based assays [e.g. using boronic acid compounds (Tan *et al.*, 2009) or cloxacillin (Brenwald *et al.*, 2005)], and rapid chromogenic assays (Livermore *et al.*, 2007). Some methods have entered commercial production [e.g. the AmpC Etest (Jacoby, 2009) and MAST ID D68C disc test (Ellem *et al.*, 2009a)], whereas others are less readily available [e.g. Syn 2190 (Doi & Paterson, 2007), Ro-48-1220 (Doi & Paterson, 2007) or LN 2-128 (Black *et al.*, 2005b) inhibitor-based assays]. The comparative sensitivity and specificity of these methods has not been comprehensively studied.

This study aimed to compare the test performance of phenotypic methods for *Bla*_{AmpC} detection in *Enterobacteriaceae* isolates from a large clinical microbiology laboratory.

METHODS

Two hundred and forty-six non-duplicate isolates, comprising 165 *K. pneumoniae*, 63 *E. coli*, 11 *P. mirabilis*, 6 *Salmonella* spp. and one *Raoultella* sp. were evaluated. The identification of all isolates was confirmed by API 20E and/or Vitek 2 (bioMérieux, France). Ninety-six per cent were clinical isolates, collected between 2006 and 2009 at PathWest Laboratories, QEII Medical Centre, Western Australia. Isolates were cultured from urine (76%), blood (8%), wound swabs (6%), sputum (4%), other sterile sites (4%) or faeces (3%). The isolates were selected to represent a diverse range of β -lactam resistance profiles, including cefoxitin resistant (77), cefoxitin susceptible (51), ESBL screening test positive/confirmatory test negative (87), confirmed ESBL (23), metallo- β -lactamase (MBL) carbapenemase (7) and *K. pneumoniae* carbapenemase (KPC) production (1: *K. pneumoniae* BAA 1705). These isolates had been characterized using either standard CLSI (CLSI, 2010) or published molecular detection methods (Senda *et al.*, 1996). Isolates were stored at -70°C in brain-heart infusion broth with 15% glycerol then subcultured twice onto Columbia agar base supplemented with 6% horse blood prior to testing. All tests were performed on Mueller-Hinton (MH) agar plates of 4 mm depth. All discs and reagents were stored at $2-8^{\circ}\text{C}$.

The phenotypic tests that were performed once each on all 246 isolates were as follows.

Cephalosporin/aztreonam susceptibility. MICs (cefoxitin, ceftriaxone, cefotaxime, ceftazidime and aztreonam) and/or inhibitory zone sizes (cefoxitin, cefotetan, cefpodoxime) were determined by agar dilution and disc diffusion respectively, as per CLSI recommendations (CLSI, 2009). MICs could not be obtained for *P. mirabilis* isolates due to the effects of swarming. Susceptibility results were interpreted according to current CLSI criteria (CLSI, 2010).

ESBL screening and confirmatory testing. As per CLSI criteria (CLSI, 2010), isolates with either MIC of $\geq 2\text{ }\mu\text{g ml}^{-1}$ (ceftazidime, cefotaxime, ceftriaxone, or aztreonam) or cefpodoxime inhibitory zone diameter $\leq 17\text{ mm}$ (or $\leq 22\text{ mm}$ for *P. mirabilis*) were considered to have a positive ESBL screen. Confirmatory testing was performed using the CLSI disc potentiation method (CLSI, 2010).

Carbapenemase testing. Isolates were screened for carbapenemase production by disc diffusion using a 10 μg meropenem disc. In accordance with CLSI recommendations, isolates with an inhibitory zone $\leq 21\text{ mm}$ had carbapenemase production confirmed using the modified (meropenem) Hodge test (CLSI, 2010).

Modified (cefoxitin) Hodge test. This was performed as described by Yong *et al.* (2002).

Tris-EDTA test. This test, also known as the 'AmpC disc test', was performed as described by Black *et al.* (2005a) (Fig. 1a).

Saline disc test. This was performed as described by Singhal *et al.* (2005).

Inhibitor-based tests. Commercially available 6 mm antibiotic discs were supplemented with either boronic acid compounds or cloxacillin, both known to inhibit *Bla*_{AmpC} activity. A 0.5 McFarland suspension of the test isolate was inoculated evenly on MH agar, and both unsupplemented and supplemented discs were applied. After incubation for 16–18 h in room air at 35°C the increase in zone size around the supplemented disc compared to the unsupplemented disc was recorded. The antibiotic substrate, inhibitor type, inhibitor concentration and proposed interpretative cut-offs vary among published methods. For the purposes of this study, all zone sizes were reviewed to establish alternative cut-offs that maximized both sensitivity and specificity for *Bla*_{AmpC} detection.

The combinations of substrate and inhibitor used were phenylboronic acid (400 μg) with either cefoxitin (30 μg) (Tan *et al.*, 2009) or cefotetan (30 μg) (Coudron, 2005), phenylboronic acid (300 μg) with either cefotaxime (30 μg) or ceftazidime (30 μg) (Yagi *et al.*, 2005), aminophenylboronic acid hemisulfate (APBA) (300 μg) with either cefotetan (30 μg) or ceftazidime (30 μg) (Tenover *et al.*, 2009), 2-benzothienylboronic acid (64 μg) with cefpodoxime (10 μg) both with and without 1 μg of clavulanic acid (Brenwald *et al.*, 2005), cloxacillin (100 μg) with cefoxitin (30 μg) (Brenwald *et al.*, 2005), and cloxacillin (200 μg) with cefoxitin (30 μg) (Tan *et al.*, 2009). Blank discs with inhibitor alone were used to detect intrinsic antimicrobial activity of the inhibitor.

MAST ID D68C disc test. A 0.5 McFarland suspension of the test isolate was inoculated evenly on MH agar, then one each of four discs (A–D) were placed onto the agar in accordance with the manufacturer's instructions (Mast Group). Disc A contained cefpodoxime (10 μg), disc B contained cefpodoxime (10 μg) and an unidentified ESBL inhibitor, disc C contained cefpodoxime (10 μg) and an unidentified AmpC inhibitor, and disc D contained cefpodoxime (10 μg) and both the AmpC and ESBL inhibitors. Incubation was for 18–24 h in room air at 35°C . A zone difference of $\geq 5\text{ mm}$ between disc C and disc A, or between disc D and disc B, was taken to indicate the isolated presence of a *Bla*_{AmpC} (Fig. 1b), whereas a zone difference of $\geq 5\text{ mm}$ between disc D and disc C, but $< 5\text{ mm}$ difference between disc B and disc A, was taken to indicate the presence of both a *Bla*_{AmpC} and an ESBL.

AmpC Etest. Double-sided Etest strips (BioMérieux) containing cefotetan ($0.5-32\text{ }\mu\text{g ml}^{-1}$) with and without cloxacillin were applied to MH agar that had been inoculated evenly with a 0.5 McFarland suspension of the test isolate in accordance with the manufacturer's

Fig. 1. (a) A positive result obtained from Bla_{AmpC} confirmatory testing using the Tris-EDTA test. This shows a zone of inhibition of *E. coli* ATCC 25922 surrounding a cefoxitin disc, with indentation adjacent to a Tris-EDTA-impregnated disc smeared with several colonies of the test isolate. (b) A positive result obtained from Bla_{AmpC} confirmatory testing using the MAST ID D68C disc test. This shows a difference in inhibitory zone size of ≥ 5 mm between discs containing cefpodoxime with (C, D) and without (A, B) an unidentified Bla_{AmpC} inhibitor.

instructions. After overnight incubation, an eightfold reduction in cefotetan MIC in the presence of cloxacillin, the presence of a 'phantom' zone of inhibition or deformation of the inhibition ellipse was interpreted as presence of a Bla_{AmpC} . The result for isolates with a cefotetan MIC $>32 \mu\text{g ml}^{-1}$ both with and without cloxacillin were considered indeterminate.

Cica-Beta chromogenic test. This test, which utilizes a chromogenic oxyminocephalosporin substrate (HMRZ-86), was performed as described by Livermore *et al.* (2007).

Three-dimensional enzyme extraction assay. This assay was performed as described by Coudron *et al.* (2000). In view of the fact that carbapenemases may hydrolyse cefoxitin, the presence of enhanced growth of organisms at the point on the agar surface where the slit intersected the zone of inhibition was only taken as evidence of Bla_{AmpC} when in combination with negative carbapenemase testing.

PCR. Detection of plasmid-borne genes encoding Bla_{AmpC} was performed based upon the method described by Pérez-Pérez & Hanson (2002). This multiplex nucleic acid amplification assay utilizes PCR primers specific for bla_{DHA} , bla_{ACC} , bla_{MOX} , bla_{CTT} , bla_{FOX} and bla_{EBC} and is capable of detecting six phylogenetic families of plasmid-mediated Bla_{AmpC} on the basis of amplicon size. All positive PCR results were confirmed by multiplex PCR using individual primer sets with a primer concentration of $0.2 \mu\text{M}$. The identity of PCR products was established by DNA sequencing performed using the Big Dye terminator system and a 3130XL instrument (Applied Biosystems), followed by a GenBank BLASTN search.

Performance and interpretation of the phenotypic testing was performed without reference to the results of PCR assays. *E. coli* ATCC 25922 (wild-type) was used as a negative control for all tests. Genotypically well-characterized Bla_{AmpC} -expressing *K. pneumoniae* served as positive controls for phenotypic testing. For PCR testing, genotypically confirmed representatives of each of six phylogenetic Bla_{AmpC} families acted as positive controls. For this purpose, an *E. coli* expressing FOX β -lactamase (J53 pMG252) was kindly provided by George Jacoby (Lahey Clinic, Burlington, MA, USA). John Iredell (ICPMR, Westmead Hospital, Sydney, Australia) kindly provided *K. pneumoniae* expressing CMY-2 (Jie091) and DHA β -lactamases (Jie142), an *Enterobacter* sp. expressing the EBC β -lactamase (W27Agr06), an *Aeromonas* sp. expressing the MOX β -lactamase (Ahc09) and a *Hafnia alvei* expressing the ACC β -lactamase (Hav09).

For the purpose of analysis, for all isolates other than *E. coli*, the reference method was detection of plasmid-encoded Bla_{AmpC} using the PCR assay. For *E. coli*, two reference methods were used for

Bla_{AmpC} detection: a positive PCR assay was interpreted as evidence of a plasmid-encoded Bla_{AmpC} , whereas the combination of a negative PCR assay, a positive three-dimensional enzyme extraction assay and a negative carbapenemase test was taken to represent hyperproduction of chromosomally encoded Bla_{AmpC} . The three-dimensional enzyme extraction assay selected for this purpose had previously been shown to have 100 % sensitivity and specificity for Bla_{AmpC} detection (Yong *et al.*, 2002).

Fisher's exact test was used to compare proportions using GraphPad Instat 3.06.

RESULTS

Seventy-four (30 %) isolates demonstrated evidence of a Bla_{AmpC} using the reference method, including 47 *E. coli*, 23 *K. pneumoniae*, 3 *P. mirabilis* and 1 *Salmonella* sp. (Table 1). Of the 47 *E. coli*, 22 (47 %) had hyperproduction of chromosomal Bla_{AmpC} and 25 (53 %) had plasmid-encoded Bla_{AmpC} . Of the study isolates with plasmid-encoded Bla_{AmpC} , the proportion with CMY (77 %), DHA (20 %) and EBC (4 %) enzymes was in keeping with the known epidemiology of plasmid-encoded Bla_{AmpC} in Australia (Ellem *et al.*, 2009b). A previously recognized (Tan *et al.*, 2009) association between certain bacterial species and Bla_{AmpC} type was observed (i.e. *K. pneumoniae* and DHA-1, *E. coli* and CMY-2).

The test performance of various proposed screening and confirmatory tests for Bla_{AmpC} detection is shown in Tables 2 and 3, respectively.

Comparison of the methods for detecting different Bla_{AmpC} families revealed that the inhibitor-based methods utilizing cloxacillin and cefoxitin were significantly less sensitive for detection of DHA enzymes compared to CMY enzymes (0 % vs 58 % for 100 μg cloxacillin, $P=0.0036$; 0 % vs 78 % for 200 μg of cloxacillin, $P<0.0001$).

Fig. 2(a, b) are histograms demonstrating the inhibitory zone sizes for cefoxitin and cefotetan as determined by disc diffusion, separated according to the presence or absence of Bla_{AmpC} . Using CLSI breakpoints, non-susceptibility to cefoxitin rather than cefotetan was a more sensitive (97 % vs 47 %, $P<0.0001$) but less specific (64 % vs 95 %, $P<0.0001$) means of screening for the presence of Bla_{AmpC} .

Table 1. Bla_{AmpC}-expressing isolates, according to bacterial species and enzyme family

Species	No. of isolates	Plasmid-encoded Bla _{AmpC} family			Hyperproduction of chromosomal Bla _{AmpC}
		CMY*	DHA†	EBC‡	
<i>E. coli</i>	47	25	0	0	22
<i>K. pneumoniae</i>	23	11	8	2	0
<i>P. mirabilis</i>	3	2	1	0	0
<i>Salmonella</i> sp.	1	1	0	0	0

*All were CMY-2, except a single CMY-14-like Bla_{AmpC} in an *E. coli* isolate.

†All were DHA-1.

‡Unable to distinguish between ACT-1/MIR-1.

Five isolates co-expressed both Bla_{AmpC} and ESBL activity. Compared to isolates that expressed only Bla_{AmpC}, the performance of detection methods for these five isolates did not differ significantly, with the exception of the inhibitor-based method utilizing cefpodoxime and 2-benzothienylboronic acid, which was less sensitive for detection of co-producers of Bla_{AmpC} and ESBL (0% vs 69%, $P=0.0159$).

AmpC Etest results for 10 (4%) of the isolates were indeterminate. Of these, six expressed carbapenemases, one an ESBL and one a Bla_{AmpC}, whilst the remainder had no identifiable β -lactamases.

When tested in isolation, none of the Bla_{AmpC} inhibitors displayed intrinsic antimicrobial activity against the study isolates.

DISCUSSION

A lack of consensus regarding optimal methods for Bla_{AmpC} detection is a major barrier to defining their

epidemiology and understanding their clinical significance (Sidjabat *et al.*, 2009). A mechanism-based approach to Bla_{AmpC} detection may reduce erroneous cephalosporin susceptibility reporting (Thomson, 2010), and enable laboratories to explain a significant proportion of false-positive ESBL screening and false-negative ESBL confirmatory tests (Robberts *et al.*, 2009). In the face of reducing development of new antibiotics, the early identification of, and application of basic infection control principles to, patients harbouring bacteria with broad-spectrum resistance mechanisms becomes paramount (Thomson, 2010). For these reasons, the development of standardized guidelines for detection of broad-spectrum β -lactamases was recently identified as a priority for both national and international laboratory method committees (Giske *et al.*, 2009).

The large number of isolates tested, the breadth of Bla_{AmpC} detection methods assessed and the inclusion of isolates possessing ESBL and carbapenemases enabled a comprehensive comparison of test performance in our study. Akin

Table 2. Test performance of proposed Bla_{AmpC} screening methods

Criterion	Method	Sensitivity	Specificity
Cefoxitin non-susceptibility*	MIC†	70/71 (99%)	73/164 (45%)
Cefoxitin non-susceptibility*	Disc diffusion	72/74 (97%)	110/172 (64%)
Cefoxitin inhibitory zone size <16 mm	Disc diffusion	68/74 (92%)	121/172 (70%)
Cefotetan non-susceptibility*	Disc diffusion	35/74 (47%)	163/172 (95%)
Cefoxitin non-susceptibility* + ceftazidime non-susceptibility*	Disc diffusion	53/74 (72%)	159/172 (92%)
Positive ESBL screen*	Disc diffusion and/or MIC	71/74 (96%)	118/172 (69%)
Positive ESBL screen* + negative ESBL confirmatory test*	Disc diffusion and/or MIC	66/74 (89%)	143/172 (83%)
Positive ESBL screen* + cefoxitin non-susceptibility*	Disc potentiation		
	Disc diffusion and/or MIC	70/74 (95%)	144/172 (84%)
	Disc diffusion		
Positive ESBL screen* + cefepime susceptibility*	Disc diffusion and/or MIC	68/71 (96%)	150/164 (91%)
	MIC†		

*As per CLSI criteria (CLSI, 2010).

†Excluding the 11 *P. mirabilis* isolates.

Table 3. Test performance of proposed Bla_{AmpC} confirmatory methods

Method	Sensitivity	Specificity
Modified (cefotaxime) Hodge test	54/74 (73 %)	163/172 (95 %)
Tris-EDTA test	72/74 (97 %)	169/172 (98 %)
Saline disc test	64/74 (86 %)	162/172 (94 %)
Phenylboronic acid (400 μ g)*		
Cefotaxime	49/74 (66 %)	168/172 (98 %)
Cefotetan	57/74 (77 %)	170/172 (99 %)
Phenylboronic acid (300 μ g)*		
Cefotaxime	33/74 (45 %)	171/172 (99 %)
Ceftazidime	58/74 (78 %)	171/172 (99 %)
APBA (300 μ g)*		
Cefotetan	51/74 (69 %)	171/172 (99 %)
Ceftazidime	58/74 (78 %)	171/172 (99 %)
2-Benzothienylboronic acid (64 μ g)*		
Cefpodoxime	45/74 (61 %)	172/172 (100 %)
Cefpodoxime + clavulanic acid	45/74 (61 %)	151/172 (88 %)
Cloxacillin (100 μ g)*		
Cefotaxime	39/74 (53 %)	171/172 (99 %)
Cloxacillin (200 μ g)*		
Cefotaxime	52/74 (70 %)	172/172 (100 %)
MAST ID D68C disc test	71/74 (96 %)	169/172 (98 %)
AmpC Etest†	62/74 (84 %)	161/172 (94 %)
Cica-Beta test	14/74 (19 %)	172/172 (100 %)

*Increase in inhibitory zone of ≥ 3 mm was the criterion for a positive result.

†10 indeterminate results were included in the analysis.

to ESBL detection, the low prevalence of Bla_{AmpC} favours a two-step process in which a highly sensitive screening test is followed by a more specific confirmatory test (Doi & Paterson, 2007). Interpretative cut-off points used in this study were selected to support a two-step evaluative test process.

Cephalexin hydrolysis distinguishes Bla_{AmpC} from many other β -lactamases. We found that non-susceptibility to cefotaxime, rather than cefotetan, was a more useful screening tool for Bla_{AmpC} detection, with a sensitivity of ≥ 97 % using either an MIC or disc-based method. The use of a cefotaxime inhibitory zone size less than the CLSI breakpoint (or a higher MIC) improved specificity with marginal loss of sensitivity (Fig. 2a, Table 2), as has been shown elsewhere (Coudron *et al.*, 2000). Other mechanisms of cephalexin resistance (e.g. porin deficiency, carbapenemases) and the fact that the cefotaxime CLSI breakpoint is only twice the modal wild-type cefotaxime MIC (Livermore, 1995) weakens the specificity of a screening approach using cefotaxime non-susceptibility. A positive ESBL screen is an adequate alternative (sensitivity 96 %) that is familiar to most laboratories. The use of combined screening tests improved specificity, but with variable impact on sensitivity. The optimal screening test combination was a positive ESBL screen plus ceftazidime susceptibility, which had a sensitivity of 96 % and specificity of 91 %.

The performance of confirmatory tests for Bla_{AmpC} detection varied widely, ranging in sensitivity from 19 % to 97 % and in specificity from 88 % to 100 %. Only two

Fig. 2. (a) Cefotaxime inhibition zone sizes for Bla_{AmpC}-containing (dark grey bars) and non-Bla_{AmpC}-containing (light grey bars) isolates. The CLSI breakpoint for non-susceptibility is <18 mm (dotted line) (CLSI, 2010). (b) Cefotetan inhibition zone sizes for Bla_{AmpC}-containing (dark grey bars) and non-Bla_{AmpC}-containing (white bars) isolates. The CLSI breakpoint for non-susceptibility is <16 mm (dotted line) (CLSI, 2010).

methods, the Tris-EDTA and MAST ID D68C disc test, had both sensitivity and specificity >90 %. The Tris-EDTA test performed better than the other biological assays, presumably because Tris-EDTA improves the release of β -lactamases by permeabilizing Gram-negative cells (Thomson, 2010). Another property of Tris-EDTA, the inhibition of MBL carbapenemase activity (Thomson, 2010), improved the specificity of this assay by preventing cefoxitin hydrolysis by this enzyme, as was demonstrated in six of the seven MBL-expressing isolates. The high sensitivity and specificity of this method has been reported in other studies (Black *et al.*, 2005a). Whilst the commercial MAST ID D68C disc test performed as well as the in-house Tris-EDTA method, lower acquisition costs may make the latter more cost effective. The AmpC Etest was easy to use, but was unsuitable for testing for Bla_{AmpC} in 4 % of isolates, although the majority of those did not possess an Bla_{AmpC}. Test performance could be improved by developing an Etest strip with a broader MIC range. The poor performance of the chromogenic Cica-Beta test is consistent with findings of other studies (Montgomery *et al.*, 2008).

Inhibitor-based assays are the most widely published Bla_{AmpC} confirmatory method. We found the sensitivity and specificity of all the inhibitor-based methods to be lower than previously described (Tan *et al.*, 2008). In contrast to other studies suggesting that cefotaxime was the optimal substrate (Hope *et al.*, 2008), we found ceftazidime to be superior, perhaps explained by preferential hydrolysis of this cephalosporin by Bla_{AmpC} (Philippon *et al.*, 2002). When used at lower concentrations, cloxacillin appeared to have inferior sensitivity compared to the boronic acid compounds. However, comparisons must be made with caution as the concentration of each inhibitor that optimizes test performance remains to be defined. When used with the optimal cephalosporin substrate, phenylboronic acid and ABPA were superior to 2-benzothienylboronic acid, but still lacked sensitivity. Notably, all the boronic-acid-based assays produced false-positive results when tested on the KPC carbapenemase-producing isolate. Boronic acid is a known inhibitor of this enzyme (Thomson, 2010). Establishing the optimal inhibitor type, inhibitor concentration, cephalosporin substrate(s) and interpretative cut-offs for this group of tests requires further research.

Variation in test performance between Bla_{AmpC} families is known to occur. For example, due to differing substrate specificity, cephamycin non-susceptibility is a less sensitive screening tool for the ACC family of enzymes (Tan *et al.*, 2009). We observed differing sensitivity of cloxacillin inhibitor-based assays for detection of CMY and DHA enzymes, which may be explained by variation in the inhibitory capacity of cloxacillin between Bla_{AmpC} families (Philippon *et al.*, 2002). These unique findings warrant further investigation on a broader range of bacterial species and Bla_{AmpC} families.

Our study has several limitations. We included relatively few isolates with co-production of both Bla_{AmpC} and ESBL.

This infrequently described combination is known to weaken the performance of inhibitor-based methods of Bla_{AmpC} detection (Tenover *et al.*, 2009), as was observed for the cefpodoxime and 2-benzothienylboronic acid combination in our study. To address this, further investigation of methods simultaneously utilizing clavulanic acid and Bla_{AmpC} inhibitors is warranted. Secondly, the geographical distribution of plasmid-encoded Bla_{AmpC} families varies widely (Alvarez *et al.*, 2004) and as we demonstrated, the performance of phenotypic tests may differ between these families, potentially restricting the applicability of our findings to other settings. However, in our study the majority of plasmid-encoded Bla_{AmpC} were of the CMY type, the most prevalent type in all other regions of the world (Jacoby, 2009). Finally, it is possible that other Bla_{AmpC} families, not detected by the multiplex PCR assay used, were present in our study. A novel plasmid-encoded Bla_{AmpC}, CFE-1, has recently been described, although its prevalence remains to be defined (Nakano *et al.*, 2004).

In conclusion, a number of Bla_{AmpC} screening options exist. These have excellent sensitivity but the performance of confirmatory tests differs widely. Further investigation of optimal substrates, inhibitory type, inhibitor concentration and interpretative cut-offs is needed in order to improve the performance of the inhibitor-based assays. A simple disc-based protocol utilizing cefoxitin non-susceptibility as a screening method, then using the Tris-EDTA method for confirmation, could conveniently be incorporated into laboratory workflow and would detect Bla_{AmpC} with a sensitivity of 95 % and a specificity of 98 %.

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REFERENCES

- Alvarez, M., Tran, J. H., Chow, N. & Jacoby, G. A. (2004). Epidemiology of conjugative plasmid-mediated AmpC beta-lactamases in the United States. *Antimicrob Agents Chemother* **48**, 533–537.
- Black, J. A., Moland, E. S. & Thomson, K. S. (2005a). AmpC disk test for detection of plasmid-mediated AmpC beta-lactamases in *Enterobacteriaceae* lacking chromosomal AmpC beta-lactamases. *J Clin Microbiol* **43**, 3110–3113.
- Black, J. A., Thomson, K. S., Buynak, J. D. & Pitout, J. D. (2005b). Evaluation of beta-lactamase inhibitors in disk tests for detection of plasmid-mediated AmpC beta-lactamases in well-characterized clinical strains of *Klebsiella* spp. *J Clin Microbiol* **43**, 4168–4171.
- Brenwald, N. P., Jevons, G., Andrews, J., Ang, L. & Fraiese, A. P. (2005). Disc methods for detecting AmpC β -lactamase-producing

- clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *J Antimicrob Chemother* **56**, 600–601.
- CLSI (2009).** *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, M7-A8. Wayne, PA: Clinical Laboratory Standards Institute.
- CLSI (2010).** *Performance Standards for Antimicrobial Susceptibility Testing*, M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute.
- Coudron, P. E. (2005).** Inhibitor-based methods for detection of plasmid-mediated AmpC beta-lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*. *J Clin Microbiol* **43**, 4163–4167.
- Coudron, P. E., Moland, E. S. & Thomson, K. S. (2000).** Occurrence and detection of AmpC β -lactamases among *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolates at a veterans medical center. *J Clin Microbiol* **38**, 1791–1796.
- Ding, H., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., Wang, A., Deng, Q., Zhang, H. & other authors (2008).** The prevalence of plasmid-mediated AmpC beta-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from five children's hospitals in China. *Eur J Clin Microbiol Infect Dis* **27**, 915–921.
- Doi, Y. & Paterson, D. L. (2007).** Detection of plasmid-mediated class C beta-lactamases. *Int J Infect Dis* **11**, 191–197.
- Ellem, J., Thomas, L., Olma, T. & Iredell, J. (2009a).** Comparison and evaluation of a newly developed Mast 4-Disc test for the detection of plasmid-mediated AmpC beta-lactamases. In *Australian Society for Antimicrobials Conference, Sydney, Australia*. Abstract no. P2.3.
- Ellem, J., Thomas, L., Olma, T. & Iredell, J. (2009b).** Rapid detection of AmpC beta-lactamases by multiplex real-time PCR and melt curve analysis. In *Australian Society for Antimicrobials Conference, Sydney, Australia*. Abstract no. P2.4.
- Giske, C. G., Sundsfjord, A. S., Kahlmeter, G., Woodford, N., Nordmann, P., Paterson, D. L., Cantón, R. & Walsh, T. R. (2009).** Redefining extended-spectrum beta-lactamases: balancing science and clinical need. *J Antimicrob Chemother* **63**, 1–4.
- Hope, R., Warner, M. & Livermore, D. M. (2008).** Phenotypic AmpC detection – which inhibitor is best? In *18th European Congresses of Clinical Microbiology and Infectious Diseases Conference, Barcelona, Spain*. Abstract no. P876.
- Jacoby, G. A. (2009).** AmpC beta-lactamases. *Clin Microbiol Rev* **22**, 161–182.
- Kohner, P. C., Robberts, F. J., Cockerill, F. R., III & Patel, R. (2009).** Cephalosporin MIC distribution of extended-spectrum-beta-lactamase- and pAmpC-producing *Escherichia coli* and *Klebsiella* species. *J Clin Microbiol* **47**, 2419–2425.
- Livermore, D. M. (1995).** β -Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev* **8**, 557–584.
- Livermore, D. M., Warner, M. & Mushtaq, S. (2007).** Evaluation of the chromogenic Cica- β -Test for detecting extended-spectrum, AmpC and metallo- β -lactamases. *J Antimicrob Chemother* **60**, 1375–1379.
- Moland, E. S., Black, J. A., Ourada, J., Reisbig, M. D., Hanson, N. D. & Thomson, K. S. (2002).** Occurrence of newer beta-lactamases in *Klebsiella pneumoniae* isolates from 24 U.S. hospitals. *Antimicrob Agents Chemother* **46**, 3837–3842.
- Montgomery, J., Nakos, J. & Gurtler, V. (2008).** Comparison of phenotypic tests for detection of plasmid-mediated AmpC beta-lactamases. In *Australian Society for Antimicrobials Conference, Sydney, Australia*. Abstract no. P2.2.
- Mulvey, M. R., Bryce, E., Boyd, D. A., Ofner-Agostini, M., Land, A. M., Simor, A. E. & Paton, S. (2005).** Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. *Antimicrob Agents Chemother* **49**, 358–365.
- Munier, G. K., Johnson, C. L., Snyder, J. W., Moland, E. S., Hanson, N. D. & Thomson, K. S. (2010).** Positive extended-spectrum-beta-lactamase (ESBL) screening results may be due to AmpC beta-lactamases more often than to ESBLs. *J Clin Microbiol* **48**, 673–674.
- Nakano, R., Okamoto, R., Nakano, Y., Kaneko, K., Okitsu, N., Hosaka, Y. & Inoue, M. (2004).** CFE-1, a novel plasmid-encoded AmpC β -lactamase with an *ampR* gene originating from *Citrobacter freundii*. *Antimicrob Agents Chemother* **48**, 1151–1158.
- Pérez-Pérez, F. J. & Hanson, N. D. (2002).** Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* **40**, 2153–2162.
- Philippon, A., Arlet, G. & Jacoby, G. A. (2002).** Plasmid-determined AmpC-type beta-lactamases. *Antimicrob Agents Chemother* **46**, 1–11.
- Robberts, F. J. L., Kohner, P. C. & Patel, R. (2009).** Unreliable extended-spectrum β -lactamase detection in the presence of plasmid-mediated AmpC in *Escherichia coli* clinical isolates. *J Clin Microbiol* **47**, 358–361.
- Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N. & Ohta, M. (1996).** PCR detection of metallo- β -lactamase gene (*bla_{IMP}*) in gram-negative rods resistant to broad-spectrum β -lactams. *J Clin Microbiol* **34**, 2909–2913.
- Sidjabat, H. E., Paterson, D. L., Qureshi, Z. A., Adams-Haduch, J. M., O'Keefe, A., Pascual, A., Rodríguez-Baño, J. & Doi, Y. (2009).** Clinical features and molecular epidemiology of CMY-type beta-lactamase-producing *Escherichia coli*. *Clin Infect Dis* **48**, 739–744.
- Singhal, S., Mathur, T., Khan, S., Upadhyay, D. J., Chugh, S., Gaiind, R. & Rattan, A. (2005).** Evaluation of methods for AmpC beta-lactamase in gram negative clinical isolates from tertiary care hospitals. *Indian J Med Microbiol* **23**, 120–124.
- Tan, T. Y., Ng, S. Y., Teo, L., Koh, Y. & Teok, C. H. (2008).** Detection of plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *J Clin Pathol* **61**, 642–644.
- Tan, T. Y., Ng, L. S., He, J., Koh, T. H. & Hsu, L. Y. (2009).** Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. *Antimicrob Agents Chemother* **53**, 146–149.
- Tenover, F. C., Emery, S. L., Spiegel, C. A., Bradford, P. A., Eells, S., Endimiani, A., Bonomo, R. A. & McGowan, J. E., Jr (2009).** Identification of plasmid-mediated AmpC beta-lactamases in *Escherichia coli*, *Klebsiella* spp., and *Proteus* species can potentially improve reporting of cephalosporin susceptibility testing results. *J Clin Microbiol* **47**, 294–299.
- Thomson, K. S. (2010).** Extended-spectrum-beta-lactamase, AmpC, and carbapenemase issues. *J Clin Microbiol* **48**, 1019–1025.
- Yagi, T., Wachino, J., Kurokawa, H., Suzuki, S., Yamane, K., Doi, Y., Shibata, N., Kato, H., Shibayama, K. & Arakawa, Y. (2005).** Practical methods using boronic acid compounds for identification of class C beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. *J Clin Microbiol* **43**, 2551–2558.
- Yong, D., Park, R., Yum, J. H., Lee, K., Choi, E. C. & Chong, Y. (2002).** Further modification of the Hodge test to screen AmpC beta-lactamase (CMY-1)-producing strains of *Escherichia coli* and *Klebsiella pneumoniae*. *J Microbiol Methods* **51**, 407–410.