

use of a highly selected collection of isolates, all of which had a low penicillin MIC according to Vitek 2. In contrast to previous studies, a molecular method was used as the reference standard. Pitkälä *et al.* [9] also used PCR detection of *bla_Z* as the reference standard, but only with bovine *S. aureus* isolates.

In conclusion, the results of this study discourage the use of nitrocefin tests or the starch–iodine plate method as additional assays for isolates with penicillin MICs of 0.06 or 0.12 mg/L according to the Vitek 2 system. Higher sensitivities were found for penicillin zone-edge determination and the cloverleaf assay, but it might be insufficient to report an isolate from a serious infection as penicillin-sensitive without performing a PCR to detect *bla_Z*.

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RESEARCH NOTE

Detection and molecular characterisation of plasmidic AmpC β -lactamases in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Dublin, Ireland

C. Roche^{1,2}, T. W. Boo², F. Walsh²
and B. Crowley¹

¹Department of Microbiology, Central Pathology Laboratory, St James's Hospital and ²Department of Clinical Microbiology, University of Dublin, Trinity College, Dublin, Ireland

ABSTRACT

This study determined the types of AmpC enzymes produced by *Klebsiella pneumoniae* isolates resistant to third-generation cephalosporins and the clonality of these isolates. The presence of AmpC enzymes was identified by cephalosporin–cloxacillin synergy tests. Genes encoding AmpC enzymes were characterised by PCR and sequencing. Pulsed-field gel electrophoresis (PFGE) was used to type the isolates. Fifteen *K. pneumoniae* isolates were positive for *bla_{AmpC}*, 13 were positive for *bla_{ACC-1}* and two were positive for *bla_{DHA-1}*. Production of the DHA-1 enzyme was inducible. The *ampR* gene was identified upstream of the *bla_{DHA-1}* gene. PFGE demonstrated the polyclonal origin of the isolates carrying *bla_{ACC-1}*.

Keywords ACC-1, AmpC β -lactamase, DHA-1, *Klebsiella pneumoniae*, resistance, typing

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Corresponding author and reprint requests: B. Crowley, Department of Microbiology, Central Pathology Laboratory, St James's Hospital, Dublin 8, Ireland
E-mail: bcrowley@stjames.ie

AmpC β -lactamases are being reported increasingly among *Klebsiella pneumoniae* isolates worldwide. However, the prevalence of AmpC-type β -lactamases among resistant *K. pneumoniae* isolates varies regionally, from 8.5% in the USA [1] to 55% in the UK [2]. Detection of AmpC-mediated resistance remains challenging, and there are currently no CLSI guidelines regarding the methodology for detection of this type of resistance. Few data are available concerning the identification and epidemiology of AmpC enzymes in *K. pneumoniae* in Ireland [2]. The present prospective study determined the prevalence and types of AmpC enzymes among *K. pneumoniae* isolates resistant to third-generation cephalosporins, as well as the epidemiology of *K. pneumoniae* isolates carrying plasmid-mediated AmpC β -lactamases.

Non-replicate isolates of *K. pneumoniae*, resistant to one or more of the third-generation cephalosporins ceftazidime, cefotaxime and/or cefpodoxime, were collected prospectively from April 2005 to March 2007. Susceptibilities were determined by disk-diffusion, by Etest (AB Biodisk, Solna, Sweden), by the agar dilution method according to CLSI guidelines [3], and by using the Vitek 2 GNI susceptibility test system (bioMérieux, Marcy l'Etoile, France) [3]. Identification of isolates was according to the Vitek GNI identification system (bioMérieux). Screening for AmpC β -lactamases was performed by measuring cefotaxime and ceftazidime MICs by Etest and Vitek 2 on both Mueller–Hinton medium and Mueller–Hinton medium containing cloxacillin 100 mg/L. A \geq two-fold reduction in the MIC in the presence of cloxacillin suggested the presence of an AmpC β -lactamase. Induction of AmpC was inferred if there was truncation in the zone around a cefotaxime 30- μ g disk adjacent to a ceftazidime 30- μ g disk.

Genes encoding acquired AmpC enzymes were identified using a multiplex PCR assay [4]. Subsequent PCR amplification and sequencing was performed using primers 5'-TTTTCTTTGTCATGCGGATT (forward) and 5'-ACAGGGCGTGCTGTAATACC (reverse) for *acc* genes, and primers 5'-ACACTGATTTCCGCTCTGCT (forward) and 5'-ACAATCGCCACCTGTTTTTC (reverse) for *dha* genes. The forward primer for detection of the *ampR* gene upstream of the *dha-1* gene was 5'-CAGGGTAAAGCGGTGAACAT. The nucleotide sequences reported in this study appear in GenBank under accession numbers EF554600 (*acc-1*) and EF633612 (*ampR* and *dha-1*).

Integron analysis was performed according to Levesque *et al.* [5].

Isolates were genotyped by pulsed-field gel electrophoresis using a CHEF DR II system (BioRad, Hemel Hempstead, UK) following digestion of intact bacterial DNA with *Xba*I [6]. The relatedness among isolates was calculated using the Dice coefficient, followed by cluster analysis using the unweighted pair-group method with arithmetic averages.

Of 500 clinical isolates of *K. pneumoniae*, 32 showed increased resistance to at least one oxyimino- β -lactam. Fifteen (48%) of these isolates were selected as possible AmpC producers. One isolate co-produced a class A extended-spectrum β -lactamase according to phenotypic tests (data not shown). The remaining 17 isolates also had phenotypes consistent with class A extended-spectrum β -lactamase production. The 15 selected isolates were shown to be AmpC-positive according to the multiplex PCR, with 13 being positive for ACC-type β -lactamases and two for DHA-type β -lactamases. The latter two isolates showed inducible resistance. Nucleotide sequencing revealed that the 13 *bla*_{ACC} genes were identical, and that there was 100% concordance with the known *K. pneumoniae bla*_{ACC-1} sequence (GenBank accession no. AJ133121). The sequences of the two *bla*_{DHA} genes were also identical and had 100% concordance with the known *bla*_{DHA-1} gene of *K. pneumoniae* (GenBank accession no. DQ478716). In addition, *ampR* was found upstream of the *bla*_{DHA-1} gene in both isolates. According to PCR results, the two isolates expressing DHA-1 and one isolate expressing ACC-1 were positive for an integron.

Susceptibility data are summarised in Table 1. All 15 isolates were susceptible to carbapenems and cefepime. High-level ceftazidime resistance was found only in the two isolates that produced DHA-1. All 15 isolates were resistant to co-amoxiclav, but most were susceptible to amikacin, gentamicin and ciprofloxacin.

Cluster analysis of the 13 isolates producing ACC-1 suggested three clusters of isolates with varying levels of relatedness: cluster 1 contained two identical isolates (7 and 9) with no band differences; cluster 2 contained two isolates (6 and 14) with four band differences; and cluster 3 contained two isolates (3 and 10) with six band differences. These three clusters were not related to each other. The remaining isolates were all unrelated.

Table 1. MICs of antimicrobial agents for AmpC-positive *Klebsiella pneumoniae* isolates

Isolate	MIC (mg/L)														
	Ctx	Ctx/Clox (100 mg/L)	Caz	Caz/Clox (100 mg/L)	Fox	Cftx	Tzp	Mem	Ipm	Etp	Fep	Tem	Cip	Mox	Gen
1	>32	>32	32	16	4	>32	32	0.064	0.125	0.064	2	8	1	0.5	64
2	16	0.25	16	0.25	>256	2	8	0.25	0.25	0.5	<1	2	2	2	1
3	>32	2	>256	12	16	>32	128	0.064	0.25	0.125	<1	8	>32	16	2
4	>32	4	>256	24	8	>32	128	0.125	0.125	0.125	<1	8	16	8	2
5	>32	8	>256	24	64	>32	256	0.064	0.25	0.25	<1	16	0.5	0.5	1
6	8	1	32	4	8	8	16	0.064	0.25	0.064	<1	8	2	2	2
7	4	2	32	4	8	8	16	0.064	0.25	0.064	<1	8	4	2	2
8	4	2	32	8	8	16	256	0.064	0.125	0.064	<1	8	2	0.5	2
9	8	1	64	4	8	8	16	0.064	0.25	0.064	<1	8	2	2	2
10	4	0.25	16	1	4	4	16	0.25	0.25	0.016	<1	4	0.5	0.5	2
11	16	4	32	8	>256	2	256	0.5	0.25	0.5	<1	16	>32	>32	2
12	>32	8	64	16	64	32	32	0.125	0.125	0.125	<1	16	16	8	1
13	32	4	64	16	16	16	128	0.064	0.125	0.125	<1	8	8	8	64
14	8	1	32	4	4	8	32	0.25	0.25	0.016	<1	4	0.5	0.5	2
15	8	2	32	4	8	8	128	0.125	0.25	0.064	<1	8	0.5	0.5	1

Gen, gentamicin; Cip, ciprofloxacin; Tzp, piperacillin-tazobactam; Ipm, imipenem; Mem, meropenem; Etp, ertapenem; Fox, ceftaxime; Cftx, ceftriaxone; Mox, moxifloxacin; Tem, temocillin; Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Clox, cloxacillin.

In conclusion, the present study identified two types of plasmidic AmpC enzymes, ACC-1 and DHA-1. While the two isolates producing DHA-1 constitute the first report of inducible plasmidic AmpC enzymes among *K. pneumoniae* isolates in Ireland, the main acquired AmpC enzyme was ACC-1. This enzyme was identified in 48% of *K. pneumoniae* isolates, compared to a frequency of 25% in a recent study in the UK and Ireland with similar selection criteria [2]. The findings are similar to other reports from France and Spain [7,8], which suggests that ACC-1 is spreading within Europe. Most (9/13) of the ACC-1-producing *K. pneumoniae* isolates were susceptible to ceftaxime, which is unusual, as ceftaxime resistance, combined with reduced susceptibility to oxyimino- β -lactams, is indicative of class C β -lactamase production [9]. *K. pneumoniae* isolates susceptible to ceftaxime and cefepime, but with decreased susceptibility to ceftazidime and/or cefotaxime, are suggestive of plasmidic AmpC production and should therefore undergo confirmatory testing. The pulsed-field gel electrophoresis data suggested that the incidence of transferable AmpC-mediated resistance within the hospital was the result of transfer of a mobile element rather than the spread of a single clone. There is a need for continuous surveillance of the prevalence and evolution of these enzymes in Ireland.

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