Detection of plasmid-mediated class C β-lactamases

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Introduction

β-Lactamases are produced by many bacterial species as a means of self-defense against β-lactam antimicrobials, such as penicillins, cephalosporins and carbapenems. There are four molecular classes of β-lactamases. Class A, C and D β-lactamases possess an active-site serine, whereas class B β-lactamases are metalloenzymes usually requiring a zinc molecule for their catalytic activities.1,2 Detection of different classes of β-lactamases in β-lactam-resistant Gram-negative bacteria is of paramount clinical importance, since it can often provide valuable information to clinicians leading to more effective and appropriate use of antimicrobials.3,4 Also, it serves as a powerful epidemiologic tool from the infection-control standpoint.5

Methods to screen for and confirm production of class A extended-spectrum β-lactamases (ESBLs) and class B metallo-β-lactamases (MBLs) have been established and utilized clinically. The methodology to detect ESBLs among Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli and Proteus mirabilis in clinical microbiology laboratories is validated and endorsed by the Clinical Laboratory Standards Institute (CLSI).6 In brief, it is composed of screening for diminished susceptibility to a third-generation cephalosporin or aztreonam followed by confirmation, which tests for the presence of an inhibitory effect by clavulanic acid, a class A β-lactamase inhibitor, against the antimicrobial activity of ceftazidime and cefotaxime. Simple methods to detect MBL...
production utilize specific inhibitors such as merceptatoctic acid or EDTA, which are tested against ceftazidime or imipenem. These detection methods for ESBLs and MBLs are available in different formats including broth microdilution, disk diffusion, and Etest.

On the other hand, methods to detect class C and D \( \beta \)-lactamas have not been as well established. In particular, class C \( \beta \)-lactamas, whose genes used to be confined to the chromosome of various Gram-negative species as \textit{ampC}, have disseminated worldwide on plasmids since the late 1980s and are now represent a substantial clinical threat. Their presence renders the bacteria resistant to most \( \beta \)-lactams including cephamycins and \( \beta \)-lactam/\( \beta \)-lactamase combinations, which are generally stable against ESBLs. Due to the lack of simple and reliable detection methods for class C \( \beta \)-lactamas that can be undertaken in clinical laboratories, their exact prevalence is unknown. Nevertheless, plasmid-mediated AmpC was found in 8.5% to 11% of ceftazidime-resistant \textit{K. pneumoniae} isolates in recent nationwide surveys performed in the USA. It is important to note that many organisms producing class C \( \beta \)-lactamas may not be resistant to broad-spectrum cephalosporins when conventional CLSI breakpoints are used. Yet, adverse clinical outcomes in patients with infections caused by organisms producing plasmid-mediated class C \( \beta \)-lactamas have been reported when these patients were treated with cephalosporins. Specifically, in this study, patients who were treated with broad-spectrum cephalosporins rather than carbapenems had higher mortality rates. The inoculum effect, well-known to ESBL-producing organisms, has been demonstrated among organisms producing some of the plasmid-mediated class C \( \beta \)-lactamas, potentially providing one explanation for the less reliable outcome when cephalosporins were used.

Of great importance is that organisms producing plasmid-mediated class C enzymes typically yield negative confirmatory tests for ESBL production by the current CLSI criteria. Therefore the susceptibilities will be reported ‘as they are’ (that is, apparently susceptible results for cephalosporins will not be changed to resistant, as is the case for ESBL producers). Hence, there has been a growing interest in developing convenient methods to detect production of plasmid-mediated class C \( \beta \)-lactamas by Gram-negative bacteria so that ‘susceptible’ results can be reported as ‘resistant’, as is the case with ESBL producers (Table 1). The most clinically important species are \textit{K. pneumoniae}, \textit{E. coli}, \textit{P. mirabilis} and \textit{Salmonella} serovars that lack chromosomal AmpC as well as \textit{E. coli} that only expresses its chromosomal AmpC at a basal level due to the lack of strong promoter activity.

Conjugation or transformation experiments are required to confirm the location of the \( \beta \)-lactamase genes on plasmids; such tests can only be undertaken in a reference laboratory and hence are not clinically useful. Detection of class C \( \beta \)-lactamase production in the species mentioned above almost always indicates exogenous origin of the genes, as they either lack or typically produce only basal amounts of AmpC, as mentioned above. Also, they are the species from which the majority of plasmid-mediated class C \( \beta \)-lactamas have been reported. The purpose of this article is to review the various phenotypic and genetic methods that have been described to detect plasmid-mediated class C \( \beta \)-lactamas.

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<thead>
<tr>
<th>Table 1</th>
<th>Rationale for detection of plasmid-mediated class C ( \beta )-lactamas</th>
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<tbody>
<tr>
<td>Increasing prevalence in the USA and probably worldwide (paucity of data on prevalence outside the USA)</td>
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<tr>
<td>Infection control opportunities for prevention of spread of important mechanisms of plasmid-mediated multi-drug resistance</td>
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<tr>
<td>Potential treatment failure with broad-spectrum cephalosporins since laboratories may report plasmid-mediated class C producers as susceptible to broad-spectrum cephalosporins using conventional CLSI breakpoints</td>
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<tr>
<td>Plasmid-mediated class C producers may appear susceptible using conventional CLSI breakpoints yet meet CLSI screening breakpoints for ESBLs. Since they will be negative by phenotypic confirmatory tests for ESBLs, they will be erroneously reported as susceptible to broad-spectrum cephalosporins</td>
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**Susceptibility patterns of class C \( \beta \)-lactamase-producing organisms**

Although genetically diverse, production of chromosomal or plasmid-mediated class C \( \beta \)-lactamas can be inferred to a certain extent from routine susceptibility testing patterns to several \( \beta \)-lactam agents. Unlike class A \( \beta \)-lactamas including ESBLs, class C \( \beta \)-lactamas are known to utilize 7\( \alpha \)-methoxy-\( \beta \)-lactamopehlorins, or cephamycins, such as cepoxitin or cefotetan, as substrates. Therefore, cefoxitin resistance in \textit{Enterobacteriaceae} combined with resistance to an oxyimino-\( \beta \)-lactamopehlorin such as cepazamide is often indicative of class C \( \beta \)-lactamase production. This method is sensitive in ruling in class C \( \beta \)-lactamase producers among \textit{K. pneumoniae} and \textit{E. coli}, but not specific since some ESBL-producing organisms may have elevated cefoxitin minimum inhibitory concentrations (MICs). Decreased production of outer membrane proteins has been demonstrated as the cause of non-susceptibility to cefoxitin in clinical isolates of \textit{K. pneumoniae} and \textit{E. coli}. \textit{AAC}-type class C \( \beta \)-lactamas, originating from the chromosome of \textit{Hafnia alvei}, are known to poorly inactivate cephamycins and are an exception to the rule of cefoxitin resistance. Also, efflux and impermeability of cefoxitin render this method less useful in lactose-non-fermenting Gram-negative species.

Commercially available \( \beta \)-lactamase inhibitors including clavulanate, sulbactam and tazobactam poorly inhibit class C \( \beta \)-lactamas, and therefore lack of inhibition when these agents are combined with \( \beta \)-lactam substrates may be suggestive of class C \( \beta \)-lactamase production, although this may occur with inhibitor-resistant TEM (IRT) \( \beta \)-lactamas belonging to class A as well. Additionally, some organisms that produce class A \( \beta \)-lactamas including ESBLs are resistant to \( \beta \)-lactam/\( \beta \)-lactamase inhibitor combinations such as ampicillin/sulbactam, amoxicillin/clavulanate and piperacillin/tazobactam. The potential reasons for this include hyper-production of ESBL, TEM-1 or SHV-1 enzymes, production of IRT \( \beta \)-lactamas derived from TEM-1, co-production of class D OXA-type enzymes, and co-production of class C \( \beta \)-lactamas, especially AmpC in the case of \textit{E. coli}.
An attempt was made to establish an algorithm to identify class C β-lactamase producers among Enterobacteriaceae with the use of available disks containing broad-spectrum cephalosporins and β-lactamase inhibitors. Though the investigators could not select definitive interpretive criteria, resistance to cefpodoxime followed by a negative inhibitory test combining clavulanate and cefpodoxime or ceftriaxone correlated with production of class C β-lactamase.

Another criterion that may be used is susceptibility to zwitterionic, or fourth-generation, cephalosporins such as cefepime and ceftiraxone. Class C β-lactamases do not hydrolyze them efficiently, and MIC values remain low unless there is concomitant production of ESBLs. A recent study of ceftazidime-resistant Enterobacter cloacae strains suggested that a cefepime MIC of less than 2 μg/ml was indicative of class C β-lactamase rather than ESBL production. However, a plasmid-mediated β-lactamase CMY-19, conferring low-grade cefepime resistance (MIC 4 μg/ml), was recently identified in K. pneumoniae. This change in substrate profile was due to an amino acid substitution in the H-10 helix region. The susceptibility profile of class C β-lactamase-producing organisms against cefepime will therefore require continuous monitoring.

None of the above methods are confirmatory, but taken together, a clinical laboratory may be able to screen for strains producing class C β-lactamases, especially among K. pneumoniae, K. oxytoca, E. coli, P. mirabilis and Salmonella serovars, which either lack or minimally express chromosomal AmpC β-lactamase, and even other species in Enterobacteriaceae producing chromosomal AmpC of various amounts (Table 2). Strains with plasmid-mediated class C β-lactamases should be resistant to both cephemycins and β-lactam/β-lactamase inhibitor combinations, although the specificity of these findings appears to be poor. This necessitates use of phenotypic confirmatory tests in such strains.

### Phenotypic confirmatory tests

#### Three-dimensional tests

Because of the paucity of specific inhibitors against class C β-lactamases, phenotypic confirmation of class C β-lactamase production has long relied on an inhibition test termed the three-dimensional test, which was originally developed to detect both ESBLs and class C β-lactamases. In this method, a slit is created in the agar next to and away from the disks at the end of the standard disk diffusion procedure for susceptibility testing. The slit is then inoculated with broth containing the test organism. After an overnight incubation, enzymatic inactivation of the antimicrobial is detected by inspecting the margin of the inhibition zone at the intersec-

<table>
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<td>Screening tests</td>
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<tr>
<td>Decreased susceptibility to expanded-spectrum cephalosporins and resistance to cefoxitin</td>
<td>• Highly sensitive but nonspecific</td>
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<tr>
<td>Decreased susceptibility to expanded-spectrum cephalosporins and lack of inhibition by β-lactamase inhibitors</td>
<td>• May rule in strains with decreased outer membrane production or chromosomal AmpC hyperproducers in E. coli.</td>
</tr>
<tr>
<td>Decreased susceptibility to expanded-spectrum cephalosporins but retained susceptibility to cefepime or ceftiraxone</td>
<td>• Highly sensitive but nonspecific</td>
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<tr>
<td>Confirmatory tests</td>
<td></td>
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<tr>
<td>Three-dimensional test</td>
<td>• May represent production of class B or D β-lactamase, IRTs, or hyperproduction of ESBLs</td>
</tr>
<tr>
<td>AmpC disk test</td>
<td>• Poorly sensitive and specific</td>
</tr>
<tr>
<td>3-Aminophenylboronic acid (APB)-based disk/microdilution test</td>
<td>• May miss co-production of ESBLs</td>
</tr>
<tr>
<td>Benzo[b]thiophene-2-boronic acid (BZBTH2B)-based disk test</td>
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<td>Multiplex PCR</td>
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IRT, inhibitor-resistant TEM; ESBL, extended-spectrum β-lactamases.
tion with the three-dimensional inoculation. Distortion of the usually circular inhibition zone is indicative of inactivation of the test drug, which occurs as a result of diffusion of β-lactamase through the agar from the organism in the three-dimensional slit.

In a study by Thomson and Sanders, when the surface of the plate was inoculated with a susceptible organism, e.g. E. coli ATCC 25922, instead of the test organism itself, and a cefoxitin disk was used, production of a class C β-lactamase, MIR-1, was visually discernable. While technically demanding, this report formed the basis for subsequent modifications of the three-dimensional test in an attempt to make the procedure simpler and more reproducible.

When the three-dimensional test was performed with crude enzyme extract in place of live bacterial inoculum to fill the slit, in combination with a cefoxitin disk, both high sensitivities and specificities were reported in detecting class C β-lactamase production among E. coli, K. pneumoniae and P. mirabilis. Despite the excellent results reported, this modification remained labor-intensive and required availability of a centrifuge as well.

This process was simplified a great deal with the AmpC disk test. In brief, an agar plate is inoculated with a lawn of E. coli ATCC 25922 and a cefoxitin disk is placed. Then, several colonies of the test organism are applied to an 'AmpC disk' containing Tris—EDTA and placed almost touching the cefoxitin disk. This modification dispensed with the enzyme extraction process since Tris—EDTA permeabilizes bacterial cells and releases β-lactamases into the external environment. A positive result is indentation or flattening of the zone of inhibition indicating enzymatic inactivation of cefoxitin. This test showed sensitivity of 100% and specificity of 90% in detecting class C β-lactamases when compared against the combination of iso-electric focusing and multiplex PCR as the gold standard. The utility of this method was confirmed by other investigators.

In a cefoxitin-agar medium-based assay, a circular well was made in the agar, which was then filled with crude enzyme of the test strain. A zone of growth of the test strain, a disk containing 64 µg of cefoxitin were placed in proximity to maintain sensitivity. Evaluation of the test increased in size in the presence of 20 µg of Ro 48-1220, when cefotetan and ceftazidime disks, which contain cephalosporin C β-lactamase-producing strains with high sensitivity and specificity. The specificity of the test decreased when non-cephalosporin cephalosporins were used as the substrates, since Ro 48-1220 is a potent inhibitor of class A enzymes as well.

Syn2190 is a 1,5-dihydroxy-4-pyridone monobactam with potent inhibitory activity against class C β-lactamases but negligible activity against class A enzymes. Its use for detection of class C enzyme production in a double-disk potentiation method has been attempted. However, interpretation of the results was difficult when disks containing Syn2190 and cefotixin were placed in proximity to maintain sensitivity. Evaluation of its use in different methods may be beneficial.

Several other β-lactam-based inhibitors with activity against class C enzymes have been synthesized, including a bridged monobactam Ro 48-1256, C3-substituted cephalosporin sulfones, oxapenem compounds, and methylidene penems, but none of them have been assessed for use for the purpose of β-lactamase detection.

Among non-β-lactam inhibitors, boronic acid compounds are among the best studied. Boronic acids were long known to have inhibitory effects against class C β-lactamases. Subsequently, benzylidine-2-boronic acid (BZBTH2B) was identified as a highly potent inhibitor of E. coli AmpC, as well as carbapenem and aza-boronic acids. BZBTH2B was used first for phenotypic detection of class C enzyme production. On an agar plate with a lawn of the test strain, a disk containing 64 µg of BZBTH2B was placed between cefotetan and ceftazidime disks, which confirmed the presence of class C β-lactamase in a Salmonella strain and several E. coli strains by expansion of inhibitory zones of the disks with substrate β-lactams facing the BZBTH2B disk. Methods using 3-aminophenylboronic acid (APB) were then developed. In the experiments, the disk potentiation test, double-disk synergy test and microdilution test were evaluated in K. pneumoniae and E. coli strains.
producing various plasmid-mediated class C enzymes. When 300 ng/mL of APB and the same interpretive criteria as those for detection of ESBLs as defined by CLSI were used,6 all three methods were shown to have excellent sensitivity and specificity.58 A combination of a cefotetan disk and a disk containing 120 mg of phenylboronic acid yielded comparable results in the disk potentiation test but the broth microdilution test was less sensitive.59 Other investigators further evaluated BZBTH2B at the same concentration for use in disk potentiation.60 Cefpodoxime was used as the substrate, and improved sensitivity was demonstrated when BZBTH2B was combined with clavulanic acid. This was likely due to the suppression of the activity of concomitantly produced ESBLs.60

Our suggested approach to phenotypic classification of clinically important β-lactamases is shown in Figure 1. All the tests used in the Figure can be performed by simple disk diffusion methods or Etests in the clinical laboratory. Depending on local prevalence data, the order may be modified, or some tests may be performed simultaneously to decrease reporting time.

Genetic and immunologic tests

Six genetically diverse families are known to exist among plasmid-mediated class C β-lactamases.61 Because of requirements for equipment, labor-intensiveness, and high costs, its use has been limited mainly to reference laboratories. However, it has the advantage of being able to detect the plasmid-mediated class C β-lactamase gene even in species carrying the chromosomal ampC gene, given that the gene on the plasmid does not derive from the same species.

An ELISA which utilizes a polyclonal antibody against CMY-2, which is a common plasmid-mediated class C β-lactamase in Salmonella serovars and E. coli, has been produced.62 It was able to detect many of the class C enzymes belonging to other families as well when higher concentrations were used.62 Its use remains within research settings at this time.

Summary

Plasmid-mediated class C β-lactamases, originally reported in the late 1980s, have now spread worldwide. Most of their structural genes are believed to have transferred from chromosomal ampC genes and disseminated subsequently. Reports on adverse clinical outcomes in patients infected with the producing organisms are emerging. They may not necessarily confer resistance to broad-spectrum cephalosporins when conventional CLSI breakpoints are used, although they may meet screening criteria for ESBLs. They typically have a negative confirmatory test for ESBLs and therefore laboratories may report AmpC producers as susceptible to broad-spectrum cephalosporins. This may have disastrous consequences if physicians erroneously use broad-spectrum cephalosporins to treat serious infections such as bacteremia. It is imperative, however, that Gram-negative bacteria producing plasmid-mediated class C β-lactamases are detected and reported adequately so that appropriate antimicrobial therapy and infection control measures can be initiated. Fortunately, several simple detection methods of class C β-lactamases have been reported recently. When ESBL production is suspected but the confirmatory tests are negative, the strains should be screened for the presence of class C β-lactamase, especially in species lacking chromosomal AmpC β-lactamase. The 'AmpC disk test' or inhibitor-based tests, most practically those based on boronic acid compounds, may be incorporated into ESBL detection algorithms upon further evaluation and verification, as more data on clinical implications of plasmid-mediated class C β-lactamases accumulate.

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