Evaluation of the chromogenic Cica-β-Test for detecting extended-spectrum, AmpC and metallo-β-lactamases

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Background: Extended-spectrum, metallo- and AmpC β-lactamases usually are sought subsequently to susceptibility testing, meaning that producers are not identified until 72 h after a clinical specimen is taken. Chromogenic tests might usefully shorten this delay, and we investigated the Cica-β-Test for this purpose.

Methods: Reference and clinical strains with known β-lactamases, or controls, were grown with a cepodoxime disc to promote conservation of resistance. The cultures were then tested with nitrocefin and with the Cica-β-Test, which examines for hydrolysis of the chromogenic oxyimino-cephalosporin HMRZ-86 with and without specific inhibitors of extended-spectrum, metallo- and AmpC β-lactamases. Results were scored, as colour changes from yellow to red, with the tester blinded to the strain identity and the mechanism(s) present.

Results: Proportions of extended-spectrum, metallo- and AmpC β-lactamase producers correctly identified by the Cica-β-Test were 85%, 77% and 72%, respectively. Such performance should be achievable if testing colonies from a primary culture plate, 24 h after a specimen was taken. Greater precision, albeit at more delay, would be achieved if results were read in conjunction with antibiogram data available 48 h after the specimen was taken. Limitations were frequent confusion of Klebsiella oxytoca hyperproducing K1 enzyme with AmpC hyperproducers, and that isolates with NMC-A or KPC carbapenemases were wrongly inferred to have AmpC enzymes.

Conclusions: The Cica-β-Test has the potential to provide useful therapeutic guidance, identifying isolates with potent β-lactamases and informing early therapy; it will also help to monitor β-lactamase epidemiology among multiresistant strains.

Keywords: β-lactamase detection, chromogenic cephalosporin, HMRZ-86

Introduction

Classical microbiology results are not available for at least 48 h after clinical specimens are taken, with this period further extended if additional investigations are done, for example, to seek extended-spectrum or metallo-β-lactamases (ESBLs/MBLs). This delay is problematic since multiresistant bacteria are increasingly prevalent in many centres and since early effective therapy is critical to minimizing mortality in severe infections.1

One response, sometimes called ‘new paradigm treatment’, is to start high-risk patients empirically on the broadest-spectrum antibiotics, typically carbapenems, then to step-down to narrow-spectrum agents, if possible, once the microbiology data become available.2 This strategy achieves better microbiological coverage and may reduce mortality,3 but leads to increased use of what were, until recently, reserve agents, with little in further reserve behind them. Moreover, step-down advice is often disregarded if the clinician sees that his or her patient is responding well to the initial treatment.

An alternative solution—potentially better for conserving antibiotic utility—lies in swifter microbiological investigation, allowing earlier tailoring of treatment. Numerous techniques potentially accelerate laboratory investigation, ranging from PCR-based identification of bacteria in the bloodstream, through the use of primary media to detect specific resistance types, to rapid chromogenic tests for resistance mechanisms. In the present study, we evaluated the Cica-β-Test, which uses a chromogenic oxyimino-cephalosporin, HMRZ-86,4,5 in combination with various inhibitors, to determine whether an isolate has an MBL, ESBL or hyper-produces its AmpC enzyme.

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Materials and methods

Test panel

The test strains comprised transconjugants or clinical isolates of *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Acinetobacter baumannii* or *Pseudomonas aeruginosa* with class A ESBLs (n = 74), MBLs (n = 26), derepressed AmpC (n = 25), hyperproduced K1 enzyme (n = 10), OXA carbapenemases (n = 10), OXA ESBLs (n = 4) or class A carbapenemases (n = 2). These resistance mechanisms had been established previously by standard phenotype and, in most cases, genetic methods. The controls (n = 79) comprised *E. coli*, *Klebsiella* spp. *Enterobacter* spp. and *P. aeruginosa* isolates lacking resistance to oxyimino-cephalosporins, with or without resistance to ampicillin and piperacillin mediated by acquired penicillinases. Fuller details are given in Tables 1 and 2 and their footnotes.

Testing strategy

Isolates were grown overnight on Mueller–Hinton agar plates (Oxoid, Basingstoke, UK) with a cefpodoxime 10 μg disc to promote and confirm retention of any oxyimino-cephalosporin resistance. They were then tested with the Cica-β-Test (manufacturer, Kanto Chemical, Tokyo, Japan; European distributor, Mast Group, Merseyside, UK), performed by a different member of staff to the one who had done the original plating. The tester was blinded to the strain identity and to all other information except for colony appearance and the presence and size of the cefpodoxime zone. Organisms were tested by putting a drop of 1 mM nitrocefin solution (BBL, Oxford, UK) onto a colony and observing for the development of a red colour within 15 min. In addition, all isolates were tested with the Cica-β-Test, which examines for the hydrolysis of a chromogenic oxyimino-cephalosporin substrate (HMRZ-86) in the presence or absence of inhibitors. The test comprised plasticized strips, each with a filter paper pad at one end, variably containing (i) no inhibitor, (ii) an unspecified inhibitor of MBLs (MBL test), identified elsewhere as sodium mercaptoacetic acid, (iii) clavulanic acid as an inhibitor of ESBLs (CVA test) and (iv) benzo-thiophene-2-boronic acid (2-2-benzothienyl boronic acid) as an inhibitor of AmpC enzymes (C test). Immediately before use, these pads were moistened with a 20 μL drop of Cica-β-Test substrate (HMRZ-86) at a concentration of 300 mg/L. Bacterial growth, taken from the overnight plate, was then smeared onto the pad surface. Single colonies were used, except with the C test, which was inoculated with three colonies. The tests were observed for 2–15 min for the development of a red colour.

Results were scored on the sequential basis described in the then package insert. Isolates giving a negative nitrocefin test were surmised to lack any significant β-lactamase activity and were not considered further; those giving a reaction with nitrocefin but not HMRZ-86 were deduced to have penicillinases but to lack any ESBL, MBL or derepressed AmpC. Isolates giving a red colour with HMRZ-86 were then scored first for inhibition of this reaction in the MBL test. If inhibition was seen, the organism was deduced to have an MBL; if not, the CVA test was considered and, if inhibition was seen, the organism was inferred to have an ESBL. If no inhibition was seen in either the MBL or CVA tests, the C test was considered and, if inhibition was seen, the isolate was deduced to hyperproduce an AmpC enzyme. If hydrolysis of HMRZ-86 was not inhibited in any test, then the isolate was deduced to have multiple or other β-lactamases.

Results and discussion

The performance of the Cica-β-Test is summarized in Table 1, showing how isolates with known mechanisms were classified; detail in relation to particular species and enzyme type is shown in Table 2 and discussed below. As summarized in Table 1, the Cica-β-Test gave 72% to 85% agreement with the reference data. This was under blind testing, as if done on colonies taken

<table>
<thead>
<tr>
<th>Mechanism inferred by Cica-β-Test</th>
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<tbody>
<tr>
<td>Reference data</td>
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<tr>
<td></td>
</tr>
<tr>
<td>MBL producers (26)</td>
</tr>
<tr>
<td>ESBL producers (74)</td>
</tr>
<tr>
<td>AmpC hyperproducers (25)</td>
</tr>
<tr>
<td>K. oxytoca, hyperproduced K1 enzyme (10)</td>
</tr>
<tr>
<td>Acinetobacter, OXA carbapenemases (10)</td>
</tr>
<tr>
<td>P. aeruginosa OXA ESBLs (4)</td>
</tr>
<tr>
<td>Enterobacteriatiae, class A carbapenemases (2)</td>
</tr>
<tr>
<td>Penicillinases (39)</td>
</tr>
<tr>
<td>Trace β-lactamase only (40)</td>
</tr>
</tbody>
</table>

*Inferred if HMRZ-86 was hydrolysed, with no inhibition in the MBL, CVA or C tests.
*Inferred if nitrocefin was hydrolysed, but not HMRZ-86.
*Inferred if neither HMRZ-86 nor nitrocefin was hydrolysed.
*For details of combinations of enzyme and organism, see Table 2.
*Five with OXA-23 enzymes, two hyperproducing OXA-51-like and three with OXA-58.
*Single examples, all PU21 transconjugants, with OXA-11, -14, -15 and -16 enzymes.
*One E. coli JM109 derivative with NMC-A enzyme and one K. pneumoniae with KPC-3.
*Nine to 10 isolates each of E. coli, K. pneumoniae, Enterobacter spp. and P. aeruginosa all with classical TEM, PSE, OXA or SHV enzymes.
*Ten isolates each of E. coli, K. pneumoniae, Enterobacter spp. and P. aeruginosa susceptible to piperacillin (MIC ≤ 8 mg/L) and, in the case of E. coli, also to ampicillin (MIC ≤ 8 mg/L), implying the absence of acquired penicillinases.
Cica-β-Test for β-lactamase identification

Table 2. Proportions of organisms with different resistance mechanisms giving the expected result with the Cica-β-Test

<table>
<thead>
<tr>
<th></th>
<th>MBL detection</th>
<th>ESBL detection</th>
<th>AmpC derepressed</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IMP</td>
<td>VIM</td>
<td>CTX-M group 1</td>
</tr>
<tr>
<td>E. coli</td>
<td>2/2</td>
<td></td>
<td>12/17</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td></td>
<td></td>
<td>5/5</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>2/3</td>
<td>1/1</td>
<td>15/15</td>
</tr>
<tr>
<td>M. morganii</td>
<td></td>
<td></td>
<td>9/10</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
<td>2/2</td>
</tr>
<tr>
<td>Total</td>
<td>13/15</td>
<td>7/11</td>
<td>27/32</td>
</tr>
<tr>
<td>Overall performance</td>
<td>20/26</td>
<td>76.9%</td>
<td>63/74 = 85.1%</td>
</tr>
</tbody>
</table>

*The fractions represent no. of isolates giving the expected result/total no. with the mechanism tested.

from a primary culture plate. Better performance would be achieved if the test was used in conjunction with susceptibility data, as an adjunct to interpretive reading.

More simply, 146/151 isolates known to have β-lactamases capable of conferring resistance to modern β-lactam antibiotics (i.e. ESBLs, MBLs, derepressed AmpC, K1 enzyme, OXA carbapenemases, class A carbapenemases or OXA ESBLs) hydrolysed HMRZ-86, whereas only 9/79 control isolates appeared to do so. These data equate to sensitivity and specificity rates of 96% and 89%, respectively, indicating a good potential to identify isolates likely to harbour potent β-lactamases.

**MBL producers**

All 26 known metallo-β-lactamase producers (Table 2) gave positive nitrocefin tests and 23/26 (88%) hydrolysed HMRZ-86, though 5 of them only weakly. The three hydrolysis of HMRZ-86 was not detected comprised two P. aeruginosa isolates with unsequenced VIM alleles and, more surprisingly, P. aeruginosa 101/1477, a well-known producer of IMP-1. Among the 23 HMRZ-86-positive isolates, 20 showed inhibition in the MBL test (i.e. the expected result), whereas just three failed to do so. One of these latter, a P. aeruginosa strain with an unsequenced VIM-type allele, showed inhibition in the CVA test acid and was mis-inferred to have an ESBL, the other two—a P. aeruginosa with an unsequenced VIM and a K. pneumoniae with an unsequenced IMP-type—showed no significant inhibition in any test and thus were mis-inferred to have ‘multiple or other’ β-lactamases.

**ESBL producers**

Seventy-four organisms with CTX-M, TEM, SHV, VEB and PER ESBLs were tested, representing numerous combinations of β-lactamases and species (Table 2), but biased towards E. coli, K. pneumoniae, and CTX-M enzymes, as clinically the most prevalent types. All the organisms hydrolysed both nitrocefin and HMRZ-86, though hydrolysis of the latter compound was weak in six cases. Three of the HMRZ-86 reactions (4%) appeared to be inhibited in the MBL test and these isolates (two K. pneumoniae with CTX-M group 9 enzymes and one with an unidentified ESBL) were mis-inferred to have MBLs. Nevertheless, fully 63/74 (85%) of the isolates showed inhibition in the CVA test and were correctly inferred to have ESBLs. Of the remaining eight isolates, two E. coli (one with a CTX-M group 9 enzyme and one with an unidentified enzyme) showed inhibition in the C test and were mis-inferred to hyperproduce AmpC, whereas six (five E. coli with CTX-M-15 and one K. pneumoniae with an unidentified ESBL) gave no clear inhibition in the C test and were inferred to have ‘multiple or other’ enzymes. Two of these latter isolates belonged to the highly prevalent UK E. coli strain A, whereas two other examples of this strain were correctly inferred to have ESBLs.

**AmpC hyperproducers**

Twenty-five chromosomal AmpC hyperproducing organisms were tested (Table 2). All were positive in nitrocefin tests and 23 (92%) also with HMRZ-86, though 7 of them only weakly so. The two isolates giving negative tests with HMRZ-86 were an E. cloacae and a C. freundii. Among the 23 isolates positive with HMRZ-86, 2 (9%) showed inhibition in the MBL test, whereas none did so in the CVA test; 18 (78%) showed inhibition in the C test, thus being correctly identified, whereas 3 (single representatives each of E. cloacae, C. freundii and M. morganii) were inferred to have ‘multiple or other’ enzymes, based on the absence of inhibition in any of the three systems.

**Other potent β-lactamases**

Strains with other potent β-lactamases were tested to ascertain whether their enzymes were confused with MBLs, ESBLs or AmpC. First, among these, were 10 Klebsiella oxytoca hyperproducing their K1 chromosomal β-lactamases. All rapidly hydrolysed nitrocefin and HMRZ-86, and none showed inhibition in the MBL detection test. However, two showed inhibition in the CVA test and were inferred to have ESBLs, whereas six more did so in the C test, being inferred to have derepressed AmpC; the remaining two showed no inhibition in any of the systems and were inferred to have ‘multiple or other’ β-lactamases (Table 1). All 10 A. baumannii with (inter alia) OXA-23, -51-like or -58 carbapenemases were positive with both nitrocefin and HMRZ-86 and none showed inhibition in any of the systems (MBL, CVA and C), correctly being inferred
to have ‘multiple or other’ β-lactamases (Table 1). Both organisms tested with class A carbapenemases (an \textit{E. coli} derivative with NMC-A enzyme and a \textit{K. pneumoniae} with KPC-3) hydrolysed both nitrocefin and HMRZ-86 and were negative for inhibition in the MBL and CVA tests; however, both showed inhibition in the C tests and were mis-identified as hyperproducing AmpC (Table 1). All four \textit{P. aeruginosa} transconjugants with OXA ESBLs (OXA-11, -14, -15 and -16) were positive in nitrocefin and HMRZ-86 tests, but one (with OXA-16) showed inhibition in the MBL test and the other three did so in the CVA test, suggesting the presence of conventional (i.e. class A) ESBLs (Table 1).

**Controls**

The first group of controls comprised 10 isolates each of \textit{E. coli, Klebsiella spp., Enterobacter spp.}, and \textit{P. aeruginosa} susceptible to piperacillin (MIC ≤8 mg/L) and, in the case of the \textit{E. coli}, also to ampicillin (MIC ≤8 mg/L). Despite their susceptibility, a surprising 90% of these organisms gave positive nitrocefin tests, though many of these reactions were weak. None was positive for hydrolysis of HMRZ-86. The second control group comprised 9 or 10 isolates each of \textit{E. coli, Klebsiella spp.}, \textit{Enterobacter spp.}, and \textit{P. aeruginosa} that were resistant to piperacillin but susceptible to oximino-cephalosporins, a profile associated with the production of acquired penicillinases. All were nitrocefin-positive, whereas nine (23%) were positive for hydrolysis of HMRZ-86, four of them only weakly so. These nine isolates comprised: (i) an \textit{E. coli} with both TEM-1 and OXA-1 enzymes, which was mis-inferred to have an ESBL, based on inhibition in the CVA test; (ii) a \textit{P. aeruginosa} strain with OXA-10 (PSE-2) enzyme mis-inferred to have an ESBL; (iii) a \textit{P. aeruginosa} with TEM-2 enzyme and two (out of five) with PSE-4 β-lactamases all mis-inferred to have MBLs; and (iv) two \textit{K. pneumoniae} with unidentified penicillinases inferred to have MBLs, and single isolates of \textit{E. cloacae} inferred to have an AmpC in one case and an ESBL in the other.

The present results are less favourable than those of a study in Israel,\(^{3}\) which found sensitivity and specificity rates for ESBL detection by the Cica-β-Test of 95.5% and 98.1%, respectively, among \textit{E. coli, Klebsiella spp.} and \textit{Proteus mirabilis}. However, (i) the study in Israel was not done blind, (ii) the species tested were unlikely to have significant levels of AmpC enzyme and (iii) the MBL and C tests were not done, removing potential sources of mis-identification. A Japanese study (with only its abstract available in English)\(^{6}\) found agreement with reference data for 13/17 ESBL producers (77%) and 8/11 isolates with MBLs (73%)—proportions similar to those found here.

Two groups of mis-categorization appeared non-random. Six of 10 \textit{K. oxytoca} isolates that hyperproduced K1 enzyme were mis-categorized as hyperproducing AmpC, based on the inhibition in the C test, which employs a boronic acid derivative. Boronic acid derivatives can be used to purify K1 enzyme, as well as AmpC types, by affinity chromatography, indicating a degree of binding and, therefore, inhibitor potential.\(^{9}\) Both strains with class A carbapenemases (KPC-3 and NMC-A) also were mis-categorized as having AmpC enzymes and it may be that these are also inhibited by boronic acids. Other cases where the Cica-β-Test failed to agree with reference data showed little association with enzyme type or host species. We therefore believe most of these disagreements reflected technical issues, most obviously variation in the amount of culture transferred to the strip and the difficulty of reading faint reactions. Performance might be improved by the better standardization of inocula, by the use of a higher concentration of HMRZ-86 (to give a stronger colour) or—as now suggested by the distributor—by the use of cultures that have been freshly removed from the incubator for the C test. Hypothetically it might also be useful to pre-expose the bacteria to the inhibitors before adding HMRZ-86 as the detection substrate, preventing the enzyme from being protected from inhibition by competing substrate (HMRZ-86) molecules. All these aspects deserve further investigation. It was proposed that reading overall patterns of inhibition would be superior to sequentially examining for inhibition in the MBL, CVA and C tests. However, 11/63 ESBL producers, correctly identified in the CVA test, also showed some degree of inhibition in the C test, suggesting that such a strategy would add confusion rather than resolve it.

Although the Cica-β-Test did not always allow definite β-lactamase identification, we conclude that it does provide useful early guidance. A positive test with HMRZ-86 for a colony from the primary culture plate, available 24 h post-sample, indicates that multiresistant Gram-negative bacteria are likely and that therapy should cover this probability. Unless an MBL is inferred, a carbapenem should be started or—if already given empirically—should be continued, as standard therapy both against ESBL producers and strains with derepressed AmpC. By contrast, a negative test with HMRZ-86 means that multiresistant bacteria are unlikely to be present and that there may be scope for step-down to the empirical therapy already being given. Such information might improve the delicate balance between the need for early effective antibiotics to severely-ill patients and the public health need to minimize unnecessary use of the most potent antibiotics. Used later, in conjunction with the susceptibility testing results, the test could be used to generate more definitive β-lactamase identification. Even this approach would save a day compared with the current common practice performing subsequent clavulanate or EDTA-based synergy tests on isolates found resistant to indicator drugs in the first cycle of susceptibility testing, and would also provide valuable information on β-lactamase epidemiology.

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**Transparency declarations**

D. M. L. is employed within the UK public sector and is influenced by HPA and NHS policies and attitudes on prescribing; he has received grants and accepted lecture and conference invites from numerous pharmaceutical companies and holds shares, in several, with these amounting to <5% of a well-diversified portfolio. He does not believe that his comments in this paper have been materially influenced by these factors, or that these interests will be materially influenced by his comments in this paper. Other authors: none to declare.
References


