

Detection of β -lactamase-mediated resistance

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β -Lactams are the most widely used antibiotics, and β -lactamases are the greatest source of resistance to them. Colorimetric tests are good, rapid, indicators of β -lactamase production and its contingent resistance in *Haemophilus*, *Moraxella* and *Neisseria* spp. They can also be applied to Gram-negative bacilli but are less useful, since the usual question is not *whether* a β -lactamase is produced but *which* β -lactamase? Accurate identification of the β -lactamases of Enterobacteriaceae demands gene or protein sequencing, but the broad type of enzyme can often be inferred from antibiotic susceptibility profile. Resistance to indicator third generation cephalosporins implies extended-spectrum β -lactamase (ESBL) production in *Escherichia coli* and *Klebsiella* spp., especially if susceptibility to ceftazidime is retained. With the emergence of CTX-M ESBLs, it is necessary to use ceftazidime as well as ceftazidime as an indicator, or to use ceftazidime. ESBL production can be confirmed with double disc synergy tests or with various commercial kits. Derepression of AmpC β -lactamases in *Enterobacter* spp. and *Citrobacter freundii* is also important and can be inferred from cross-resistance encompassing β -lactamase inhibitor combinations as well as all cephalosporins except fourth-generation agents. Antagonism between ceftazidime and ceftazidime implies the presence of inducible AmpC enzymes in e.g. *Enterobacter* spp., indicating the risk of segregation of derepressed mutants. In general, though, this risk is better predicted from accurate species identification. Metallo-carbapenemases are slowly emerging and present a new detection challenge, firstly because carbapenem resistance does not always accompany enzyme production and secondly because EDTA, which is used as an inhibitor in some diagnostic tests, may also exert other effects on the cell.

Introduction

β -Lactamases are the main cause of bacterial resistance to penicillins and cephalosporins. Definitive identification of these enzyme is only possible by gene or protein sequencing;¹ aspects beyond the scope of diagnostic laboratories or of this review. Nevertheless, simple β -lactamase detection and typing tests can be valuable in the clinical laboratory. These include: (i) direct tests for β -lactamase activity in fastidious Gram-negative species; (ii) tests for extended β -lactamases (ESBLs); and (iii) tests for inducibility of chromosomal β -lactamases. Tests for metallo-carbapenemases are being developed and may become increasingly useful if these enzymes spread in the future. In the longer term, gene chip technology may allow precise routine identification of β -lactamases.²

Tests for detection of β -lactamases

Direct tests for β -lactamase activity

Direct β -lactamase tests are mostly used for *Haemophilus influenzae*, *Moraxella catarrhalis* and *Neisseria* spp., where few different enzyme types occur, and where enzyme production has clear implications for therapy. Direct tests can be applied to other species, but are less useful, since the important question usually is not whether a β -lactamase is produced but *which* β -lactamase.

Numerous β -lactamase detection tests have been devised¹ but few are convenient for routine use. Most use chromogenic cephalosporins, or link the hydrolysis of penicillin to a colour change mediated by iodine or a pH indicator. Chromogenic cephalosporins are very specific, whereas acidification and the reduction of iodine can occur for reasons other than β -lactamase action, potentially giving false-positive results. Positive and negative controls should be run in parallel with all tests but, because of the risk of false-positive results, are especially critical for the acidimetric and iodometric methods.

Nitrocefin test.

Nitrocefin is a chromogenic cephalosporin that changes from yellow to red on hydrolysis. It provides the most sensitive test for most β -lactamases, exceptions being staphylococcal penicillinase and ROB-1, an uncommon plasmid-mediated enzyme of haemophili.³ Nitrocefin is available as pure powder from Becton Dickinson (Oxford, UK). Powder can also be obtained in commercial preparations, (e.g. Oxoid, Basingstoke, UK), where it is supplied together with a vial of diluent and is mixed with materials that facilitate solubilization. Various commercial devices based on nitrocefin are also available (e.g. from Oxoid and Becton Dickinson). The method described below is for pure powder; users of other preparations and devices should follow the instructions provided with them. Another chromogenic cephalosporin, PADAC,⁴ exists but is less sensitive and is not readily available.

A 0.5 mM nitrocefin solution is prepared by dissolving 2.58 mg of powder in 0.5 mL of dimethylsulphoxide (DMSO) then diluting with 9.5 mL of 0.1 M phosphate buffer, pH 7.0. This solution is stable for 10 days at 4°C in a foil-wrapped bottle. Glass containers should be used, since DMSO degrades plastics. Colonies of the test isolates are scraped from nutrient agar plates and are suspended in 20 μ L volumes of 0.1 M phosphate buffer pH 7.0, to produce a dense suspension on a glass slide, and 20 μ L amounts of the nitrocefin solution are added. β -Lactamase activity is indicated by a red colour within 1-2 min. Weak activities may take longer to appear, but reactions taking >10 min should be treated with scepticism, as they may reflect the secondary β -lactamase activity of those penicillin-binding proteins that form unstable acyl complexes.

Iodometric tests.

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolourising starch-iodine complex. This reaction can be exploited to detect β -lactamase activity in tubes or on paper strips. These tests are particularly sensitive for staphylococcal penicillinase, but are less sensitive than nitrocefin for most of the β -lactamases from Gram-negative bacteria.

(i) Tube method.

Benzylpenicillin, 6 g/L in 0.1 M phosphate buffer pH 6.0, is distributed in 0.1 mL quantities in tubes or a microtitre tray.⁵ Bacterial growth from agar (not broth) is suspended in these solutions until they are heavily turbid (c. 10^9 cfu/mL). The suspensions are held at room temperature for 30-60 min, then 20 μ L volumes of 1% (w/v) soluble starch in distilled water are added, followed by 20 μ L of 2% (w/v) iodine in 53% (w/v) aqueous potassium iodide. β -lactamase activity is indicated by decolourisation of the iodine

within 5 min. Positive and negative controls are vital, as extraneous protein reduces iodine, and over-heavily inoculated tests may give false-positive results.

(ii) Paper strip method.

To prepare iodometric paper strips, 0.2 g of soluble starch is added to 100 mL of distilled water and dissolved by boiling.⁶ After cooling, 1 g of benzylpenicillin is added. Filter papers (1 x 5 cm, Whatman No. 3; Whatman, Maidstone, UK, or similar) are soaked in this solution, then air-dried for 2 h. The strips, which are stable for 1 year at -20°C, are moistened with 2% (w/v) iodine in 53% (w/v) aqueous potassium iodide before use. They are then smeared with colonies from an overnight culture plate. Decolourisation within 5 min indicates β -lactamase activity. Positive and negative controls are mandatory.

Acidimetric tests.

Hydrolysis of the β -lactam ring generates a carboxyl group, acidifying un-buffered systems. The resulting acidity can be tested in tubes or on filter papers. The method is useful for tests on *H. influenzae* and *Neisseria gonorrhoeae*.

(i) Tube method.

For the tube method, 2 mL of 0.5% (w/v) aqueous phenol red solution is diluted with 16.6 mL distilled water and 1.2 g of benzylpenicillin is added. The pH is adjusted to 8.5 with 1 M NaOH. The resulting solution, which should be violet in colour, can be stored at -20°C. Before use, 100 μ l portions are distributed into tubes or microtitre wells and inoculated with bacteria from culture plates (not broth) to produce dense suspensions. A yellow colour within 5 min indicates β -lactamase activity. Positive and negative controls must be run in parallel.

(ii) Paper strip method.

For the acidimetric paper method,⁷ filter paper (Whatman No. 1) is cut into 5 x 1 cm strips and soaked in a freshly-prepared solution containing 125 g/L benzylpenicillin, 0.1% (w/v) bromocresol purple and 1.25 mM NaOH. The strips are dried and can be stored at 4°C for 6 months with a silica gel desiccant. Such strips are available commercially [e.g. 'IntraLactam' (Mast Diagnostics, Bootle, UK) or 'Beta-Test' (Medical Wire and Equipment, Corsham, UK)]. Before use the strips must be moistened: distilled water is recommended but it is essential that the water is not acidic, and calcareous tap water may be preferable.⁸ Bacteria from agar (not broth) cultures are smeared on the strip and development of a yellow colour within 5 min indicates β -lactamase activity. Controls must be run in parallel.

Microbiological tests of β -lactamase activity.

β -Lactamase activity can be detected biologically by demonstrating the loss of activity of a β -lactam agent against a susceptible indicator organism. There are several variations, including the cloverleaf (Hodge) method, which is highly sensitive for staphylococci, and the Masuda double disc method, which can be used with whole cells or cell extracts of test strains. While the use of such methods has declined, they remain very sensitive. A review was provided by Livermore & Williams.¹

Tests for ESBLs

ESBLs are of increasing concern.⁸ Most, worldwide, are mutants of classical TEM and SHV plasmid-mediated types but the CTX-M types, which are derived from the chromosomal β -lactamases of *Kluyvera* spp., are predominant in parts of South America⁹ and are spreading in Asia and Europe,¹⁰ with reports now from most UK laboratories.^{11,12}

Unlike the classical TEM- and SHV-1 enzymes, ESBLs hydrolyse oxymino-aminothiazolyl cephalosporins such as cefuroxime, cefotaxime, ceftriaxone, ceftizoxime, ceftazidime, cefpirome and cefepime, as well as penicillins and other cephalosporins except cephamycins. Some SHV- and TEM-derived ESBLs (e.g. SHV-5 and TEM-3) are similarly active against cefotaxime and ceftazidime but other prevalent types (e.g. TEM-10 and -26) are much more active against ceftazidime than cefotaxime or ceftriaxone.¹³ Conversely, most CTX-M types are preferentially active against cefotaxime and ceftriaxone and are less active against ceftazidime.

TEM and SHV ESBLs are most prevalent in klebsiellae, especially in intensive care and other specialized units, and are increasingly scattered also in other Enterobacteriaceae; CTX-M enzymes are found in various Enterobacteriaceae, but predominantly *Escherichia coli*, sometimes from the

community.¹¹ Several ESBLs have also been found in *Pseudomonas aeruginosa*, but many of these are unusual PER and OXA enzyme types and none is prevalent in the species outside Turkey.¹⁴

ESBL-mediated resistance to cephalosporins is not always obvious in disc or dilution tests as the MICs of cephalosporins for producers can be as low as 0.5 mg/L and the inhibition zones of discs are correspondingly large.¹ Nevertheless, even such weak ESBL activities have been associated with clinical failure in patients and in experimental animals,^{15,17} underscoring the need for accurate laboratory detection.

Various tests have been developed. Most are tailored to detect ESBLs in *Klebsiella* spp., but are equally applicable to other Enterobacteriaceae with little or no chromosomal β -lactamase activity, such as *E. coli*, *Salmonella* spp. and *Proteus mirabilis*. The detection of ESBLs in species with inducible chromosomal β -lactamases, such as enterobacters and *P. aeruginosa*, is a greater challenge.

Inference of ESBL production from cephalosporin resistance.

ESBLs are the commonest cause of resistance to oxyimino-aminothiazolyl cephalosporins in klebsiellae and *E. coli* in the UK.^{1,8} In order to detect ESBL producers, ceftazidime should be included in all first-line susceptibility testing against isolates of these species, and production should be suspected in those isolates found resistant. Cefotaxime should also be tested as the best indicator of CTX-M type enzymes. Alternatively, cefpodoxime can be tested as a single indicator of ESBLs including both CTX-M and TEM/SHV types.

ESBL production in isolates found resistant to ceftazidime, cefotaxime or cefpodoxime can then be confirmed with the double disc or commercial tests outlined below. Other oxyimino-aminothiazolyl cephalosporins are less reliable as primary indicators of ESBL production than ceftazidime and cefotaxime (or cefpodoxime) and it should particularly be noted that producers often appear to be susceptible to cefuroxime.⁸ Thus, the frequent UK practice of including this drug in first-line tests and only testing any other cephalosporin (e.g. cefotaxime and ceftazidime) as second-line agents risks failure to detect ESBL producers.

An alternative strategy detection, of testing cephalixin first line, and then to testing the resistant isolates as potential ESBL producers has proved unreliable, with some CTX-M-15 producing *E. coli*, belonging to a major UK epidemic strain appear to be susceptible. This approach is not, therefore to be recommended.

Not all third-generation cephalosporin-resistant klebsiellae and *E. coli* are ESBL producers. Some klebsiellae have acquired plasmids encoding AmpC enzymes that have 'escaped' from the chromosomes of other Enterobacteriaceae.¹⁸ Such plasmids have reached *E. coli* too, where AmpC hyper-production can also arise via chromosomal reorganisation.⁸ In the case of *Klebsiella oxytoca* (**NOT** *Klebsiella pneumoniae*), hyper-production of chromosomal K1 (KOXY) β -lactamase can arise via mutation.¹⁹ AmpC and hyperproduced K1 enzymes do not give positive results in the ESBL confirmatory tests outlined below (except that weak false positives may occur in cefotaxime/clavulanate tests with *K. oxytoca* hyper-producing K1 enzyme). Further distinguishing features are that AmpC enzymes, unlike ESBLs, confer ceftazidime resistance, and that hyperproduction of K1 enzyme characteristically confers obvious resistance to cefuroxime, aztreonam, and (usually) ceftriaxone and cefpodoxime, but not ceftazidime, while cefotaxime results are variable among strains.

The resistance patterns associated with ESBLs, plasmid AmpC enzymes and hyperproduction of K1 enzyme are summarized in the Table, and a fuller account of relationships between these (and other) resistance mechanisms and antibiogram is given in this supplement by Livermore *et al.*²⁰

Confirmatory tests for ESBLs in klebsiellae and E. coli

(i) Double disc tests.

A plate is inoculated with the test isolate, as for a routine British Society for Antimicrobial Chemotherapy (BSAC) disc susceptibility test. Discs containing cefotaxime and ceftazidime 30 μ g are applied to the medium either side of one containing co-amoxiclav 20+10 μ g; and 25-30 mm away from it.²¹ The plate is incubated overnight at 37°C, and ESBL production is inferred when the zone of either cephalosporin is expanded by the clavulanate (Figure 1). For CTX-M types, the synergy is more obvious with cefotaxime whilst, for wume TEM and SHV variants, it is more obvious with ceftazidime. The advantage of this simple method is its low cost; the disadvantage is that the optimal separation of the discs may vary with the individual strain. Consequently isolates giving equivocal results may need to be re-tested

with the discs closer together. Double disc tests between cefpodoxime and co-amoxiclav discs can be performed similarly, and detect both the TEM/SHV and CTX-M type enzymes.

(ii) Etest ESBL strips.

Etests (AB Biodisk, Solna, Sweden; Bio-Stat, Stockport, UK) with a ceftazidime or cefotaxime gradient at one end and a ceftazidime or cefotaxime plus clavulanate gradient at the other can be used to detect ESBLs (Figure 2).²² They should be used in accordance with the manufacturer's package insert, which specifies a heavier inoculum than for the BSAC disc test method. ESBL production is inferred if the ratio of the MIC of cephalosporin alone to the MIC of cephalosporin plus clavulanate is eight or more. Etests containing a cefepime/clavulanate gradient are also available and the manufacturer suggests that these are suitable for detecting all ESBLs, though there is only limited supportive data, particularly for strains with very weak ESBLs, such as TEM-12. There is also (as with cefotaxime/clavulanate tests) a risk of false-positive results for *K. oxytoca* strains hyperproducing K1 enzyme (Potz *et al.*, in press).

(iii) Combined disc methods.

These depend on comparing the zones given by discs containing an extended-spectrum cephalosporin with clavulanate with those for identical discs without this inhibitor. If an ESBL is present, the zones are enlarged by the presence of the inhibitor. The United States Clinical Laboratory Standards Institute (CLSI; formerly NCCLS) recommends comparison of the zones given by cefotaxime 30 µg versus cefotaxime + clavulanate 30 + 10 µg and ceftazidime 30 µg versus ceftazidime + clavulanate 30 + 10 µg discs.²³ These disc types are now available commercially (Oxoid or Becton Dickinson 'Combination Discs' and Mast 'MAST DD'). M'Zali *et al.*²⁴ evaluated the MAST DD in CLSI-type tests, and detection of 93% of ESBL producers (based on a 50% enlargement in zone diameters in the presence of clavulanate) if both the ceftazidime and cefotaxime disc pairs were used, whereas use of ceftazidime or cefotaxime DD alone achieved 86 and 66% sensitivity, respectively. Another combination disc method (Oxoid) depends on comparing the zones given by cefpodoxime 10 µg and cefpodoxime + clavulanate 10 + 1 µg discs. ESBL production is inferred if the zones given by the discs with clavulanate are ≥5 mm larger than those without the inhibitor.²⁵ This method was validated with both BSAC and CLSI methodology and was found to detect ESBL-producing klebsiellae with 100% sensitivity and specificity in either case.²⁵ The method distinguished klebsiellae with TEM and SHV ESBLs, which gave zones enlarged by ≥5 mm by the clavulanate, from those with AmpC or K1 enzyme, where the clavulanate gave little or no expansion of the inhibition zones.

(iv) Automated susceptibility testing systems.²

These are beyond the scope of this paper. However, it should be noted that ESBL detection tests are incorporated into standard test for Vitek and Vitek 2 (bioMérieux, Marcy l'Etoile, France) and the panels for the Phoenix (Becton Dickinson). The Vitek 2 differs in that, rather than incorporating a specific ESBL test, it best-fits the patterns of MICs profiles for the isolate to those of a reference database of MICs for isolates with known mechanisms of resistance, including ESBLs. The Vitek ESBL test was validated by Sanders *et al.* with good results.²⁶ Validation of the Advanced Expert System on the Vitek 2 system indicated 100% accuracy in detection of TEM and SHV-type ESBLs in klebsiellae.²⁷ CTX-M producers were not included, but should be detected since cefotaxime is included on the test panel. The Phoenix system achieved 100% sensitivity and 98.5% specificity in ESBL detection.²⁸ Producers of CTX-M enzymes were recognised, as well as those with TEM and SHV-derived enzymes, the only errors being a few K1 β-lactamase hyperproducers that were misidentified as ESBL-positive.

Controls for ESBL disc tests

Quality Control of the cefpodoxime, cefotaxime and/or ceftazidime discs used in primary screening should be in accordance with standard BSAC recommendations. In addition, positive controls should be used to ensure the performance of ESBL confirmatory test and, for this purpose, *E. coli* strains with the following ESBLs are available from the NCTC: CTX-M-15 (cefotaximase) NCTC 13353, TEM=3 (broad-spectrum) NCTC 13351 and TEM-10 (Ceftazidimase) NCTC 13352. In addition, when using combination discs for ESBL confirmation tests, either *E. coli* NCTC 10418 or ATCC 25922 should be used as a negative control, giving inconsistent zone diameters (±2 mm) irrespective of the presence of clavulanate in the disc. Any greater difference implies disc malfunction or deterioration and, in this regard, particular care is needed if the clavulanate containing and non-containing discs are from different batches.

Susceptibility reporting for isolates with ESBLs.

There is some evidence that beta-lactam agents might be used to treat infections with ESBL-producers as long as the MIC is below the (low) breakpoints calculated using pharmacodynamic principles, but such breakpoints are not yet clearly established and we currently recommend a cautionary approach, i.e. organisms inferred to have ESBLs should be assumed to be resistant to all penicillins, except temocillin, and all cephalosporins, except cephamycins, irrespective of the results of routine susceptibility tests. Carbapenems remain active; results for β -lactamase inhibitor combinations vary with the strain, but probably should be accepted at face value.

Detection of ESBLs in organisms with inducible AmpC.

ESBLs are more difficult to detect in those Enterobacteriaceae that typically have inducible AmpC chromosomal enzymes (e.g. *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp. and *Serratia* spp.). In these cases, the enzymes may be induced by clavulanate (which inhibits them poorly) and may then attack the indicator cephalosporin, thus masking any synergy arising from inhibition of an ESBL.⁸

P. aeruginosa presents further difficulties, in having not only an inducible AmpC enzyme but also in having a much greater degree of impermeability than Enterobacteriaceae, as well as efflux-mediated resistance.⁸

Resistance to oxymino-aminothiazolyl cephalosporins (e.g. ceftazidime, cefotaxime and cefpodoxime) more often depends on mutational hyper-production of chromosomal AmpC enzymes than on ESBL production by *Enterobacter* spp., *C. freundii*, *M. morganii*, *Providencia* spp. and *Serratia* spp.^{1,8} Nevertheless, enterobacter isolates with ESBLs are increasingly seen, and one *Enterobacter aerogenes* strain with TEM-24 β -lactamase has spread widely among hospitals in Belgium and France,^{29,30} though not yet to the UK.

Cefepime/clavulanate synergy tests, performed by a double disc method, or using Etest combination strips, are useful for detecting ESBLs in AmpC-inducible species. Cefepime is more stable than third-generation cephalosporins to AmpC activity, meaning that any clavulanate-effected induction of this enzyme is less prone to disrupt the test. Cefepime/clavulanate synergy tests are satisfactory for the epidemic TEM-24 positive *E. aerogenes* strain, but remain to be validated for enterobacters with batteries of different ESBLs, including those with only weak activity against cefepime (e.g. TEM-12).

Another helpful strategy is to test cefotetan and piperacillin/tazobactam against *Enterobacter* spp. and *C. freundii* isolates that are resistant to third-generation cephalosporins. Those isolates that are susceptible to these agents are most likely to have ESBLs, whereas those that are resistant are more likely to have derepressed AmpC.²⁰ The test is not, however, definitive and some ESBL-positive enterobacters are resistant to cefotetan and piperacillin/tazobactam. A further complication is that some strains have both ESBLs and derepression of AmpC.

In the case of *P. aeruginosa*, the best rule seems to be that ESBLs give very high levels of ceftazidime resistance (MIC >64 mg/L and growth up to the edge of a ceftazidime 30 μ g disc) whereas hyper-production of AmpC and/or impermeability/efflux function gives lower-level resistance. Clavulanate synergy tests are unreliable for ESBLs in *P. aeruginosa*, as they will fail to detect the OXA-variants, which are among the major ESBL types in the species.¹⁴ Clavulanate-based synergy tests also are unsuitable for *Acinetobacter* spp., which are often susceptible to the antibiotic activity of β -lactamase inhibitors. Cephalosporin resistance is widespread in *Acinetobacter* spp., but rarely involves the ESBLs found in other species.

Tests for inducibility of chromosomal AmpC β -lactamases

AmpC enzymes are chromosomal and inducible in most *Enterobacter* spp., *C. freundii*, *Serratia* spp., *M. morganii*, *Providencia* spp. and *P. aeruginosa*.⁸ First-generation cephalosporins, ampicillin and amoxicillin induce these enzymes and are destroyed by them. Consequently the AmpC-inducible species generally are resistant.^{1,8} Third-generation cephalosporins, cefuroxime, piperacillin and aztreonam also are labile, but induce weakly. Consequently, in the absence of other mechanisms, β -lactamase-inducible strains appear susceptible. However, these AmpC-inducible organisms segregate "derepressed" mutants, which produce their AmpC enzymes copiously without induction.^{1,8} These mutants are resistant to almost all penicillins and cephalosporins²⁰ and may overgrow during cephalosporin therapy and, having been selected in one patient, may spread to others.

Strains that are already depressed are frequently isolated, and their cephalosporin resistance is obvious, e.g. in the BSAC disc diffusion test. The problem is that AmpC-inducible organisms may be isolated from a patient and found to be susceptible to third generation cephalosporins. However, when these drugs are used clinically, there is selection of derepressed resistant mutants, with contingent treatment failure. This risk is as high as 20% in *Enterobacter* spp. bacteraemia.^{31,32} It is therefore recommended that laboratory reports should warn clinicians against the use of third-generation cephalosporins in infections caused by these and other AmpC-inducible species, except when the infection is in the urinary tract, where very high cephalosporins concentrations can be attained. No such recommendation is made for *P. aeruginosa* because, unlike AmpC-inducible Enterobacteriaceae, this species readily mutates to become resistant to alternative drugs, e.g. carbapenems and fluoroquinolones.³³

The best means of recognising AmpC-inducible Enterobacteriaceae is accurate species identification, and the common UK practice of 'lumping' these organisms as 'coliforms' is undesirable in this context. If species identification is not undertaken, most AmpC-inducible *Enterobacter* spp. and *C. freundii* (not *M. morgani*, *Providencia* spp. and *Serratia* spp.) can be recognised by their resistance to cefoxitin, but apparently susceptibility to third-generation cephalosporins, whereas species without significant levels of AmpC enzyme (e.g. *E. coli* and *Klebsiella* spp.) are susceptible to cefoxitin.³⁴ Alternatively, AmpC-inducible species can be recognised in cefoxitin/cefotaxime disc antagonism tests.³⁵ For these, plates are inoculated as for a BSAC disc test and cefotaxime 30 µg and cefoxitin 30 µg discs are placed 25 mm apart. β-lactamase inducibility is recognised because of blunting of the cefotaxime zone adjacent to the cefoxitin disc (Figure 3). Any cefoxitin-resistant, cefotaxime-susceptible (i.e. typical) *Enterobacter* spp. isolate will serve as a control, though it should be cautioned that the optimum spacing of the discs can vary with the test strain and that a successful control test may give a false sense of security without proving that the test has been optimised for the particular isolates(s) under investigation.

Class A β-lactamases (not AmpC types) are inducible in *Proteus vulgaris* and *Citrobacter diversus*. Unlike AmpC types these are inhibited by clavulanic acid.^{8,20} Selection of derepressed mutants is not a significant clinical problem in these species, and warnings about cephalosporin use do not seem warranted.¹

Acquired metallo-β-lactamases

Though still rare and predominantly reported from the Far East, acquired metallo-β-lactamases belonging to the IMP and VIM families are an increasing concern, principally in non-fermenters of the general *Pseudomonas* and *Acinetobacter*.^{36,37} They have also been reported from Enterobacteriaceae, though more rarely.

Recommended detection methods involve testing for synergy between imipenem and EDTA. These can be done using Etest combination strips, with an eight-fold MIC reduction in the presence of EDTA being taken to indicate metallo-β-lactamase production. Alternatively, synergy can be sought by placing a home-made disc containing 750 µg EDTA 20 mm away from one containing imipenem 10 µg.³⁸ Expansion of the imipenem zone adjacent to the EDTA disc implies metallo-β-lactamase production.

The problems with these methods are two-fold. First, EDTA may potentiate imipenem for reasons unconnected with inhibition of metallo-β-lactamases, perhaps because it permeabilises the bacterial cell or because zinc (which EDTA sequesters) potentiates the chemical breakdown of imipenem³⁹ or, in the case of *P. aeruginosa*, because zinc affects the regulation of the OprD porin, which facilitates the entry of imipenem.⁴⁰ Secondly, some metallo-β-lactamase producers remain almost fully susceptible to carbapenems *in vitro*, perhaps because frank resistance, especially among the Enterobacteriaceae, requires impermeability as well as the enzyme.^{41,42}

The first problem, which leads to a proportion of false-positive results in EDTA synergy tests, might be overcome by using an alternative metallo-β-lactamase inhibitor, and the second by combining EDTA with ceftazidime (to which IMP and VIM producers are more consistently resistant). However, these strategies remain to be validated. Alternative inhibitors include o-phenanthroline⁴³ and thiol compounds,⁴⁴ though Health and Safety issues preclude routine use of the latter. None of these methods would detect carbapenemases other than metallo-enzymes (e.g. OXA, SME or KPC types). Owing to these limitations, current UK advice is that all carbapenem-resistant Enterobacteriaceae and

Acinetobacter spp. should be sent for reference laboratory confirmation and investigation, whereas imipenem-resistant *P. aeruginosa* should be screened with the EDTA-based test described above, and those giving positive results should be submitted for confirmation. Because of these vagaries and because reference investigation is advised we are not, as yet, advising on any control strain for use in general diagnostic laboratories.

Conclusion

It is striking how much this chapter has had to be up-dated, only three and a half years after it was first written. This revision was not to correct errors, but to adapt to changes, principally the emergence of CTX-M and metallo- β -lactamases in the UK. These new enzymes present major challenges to the laboratory as well as to the clinician. Beyond them, perhaps the only certainty is that β -lactamase evolution is continuing...

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.Legends to Figures

Figure 1. Detection of ESBL production in double disc tests. Discs: left, ceftazidime, 30 µg; centre, amoxicillin + clavulanate 20 + 10 µg; and right, cefotaxime 30 µg. The organism is *E. coli* with TEM-5 enzyme.

Figure 2. Detection of ESBL production with an Etest ESBL. The strip has a ceftazidime gradient at both ends, with clavulanate in the right hand gradient only. The ceftazidime MIC against the *E. coli*, which produced TEM-5, is >32 mg/L in the absence of clavulanate and 0.75 mg/L in its presence. As the ratio of ceftazidime MIC with and without clavulanate is ≥ 8 the strain is inferred to be an ESBL producer.

Figure 3. Detection of an inducible AmpC enzyme in *E. cloacae*. The disc with the large zone is ceftazidime 30 µg; that with the very small zone is cefoxitin 30 µg. Note the blunting of the ceftazidime zone by the cefoxitin-induced enzyme.

Figure 4. Detection of metallo-β-lactamases in *P. aeruginosa* with metallo-β-lactamase Etests. All three panels indicate positive results, with greater sensitivity in the presence of EDTA (top of illustration) than in its absence (bottom).

Table. Antibiograms of klebsiellae and *E. coli* with ESBLs and other potent β -lactamase types

Enzyme	Antimicrobial susceptibility								Synergy with clavulanate		
	CAZ	CTX	CTR	CXM	CPD	FOX	AZT	IMP/MEM	CAZ	CTX	CPD
ESBL	R	v	v	v	R	S	v	S	+	+	+
AmpC	R	R	R	R	R	R	R	S	-	-	-
K1, <i>K. oxytoca</i> only	S	v	v/R	R	R	S	R	S	-	-	-
CTX-M	v	R	R	R	R	S	v	S	+/-	+	+

S, susceptible; v, variable with type and amount of enzyme; R, resistant; AZT, aztreonam; CAZ, ceftazidime; CPD, cefpodoxime; CTR, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; FOX, ceftoxitin; IMP, imipenem; MEM, meropenem.

Figure 1

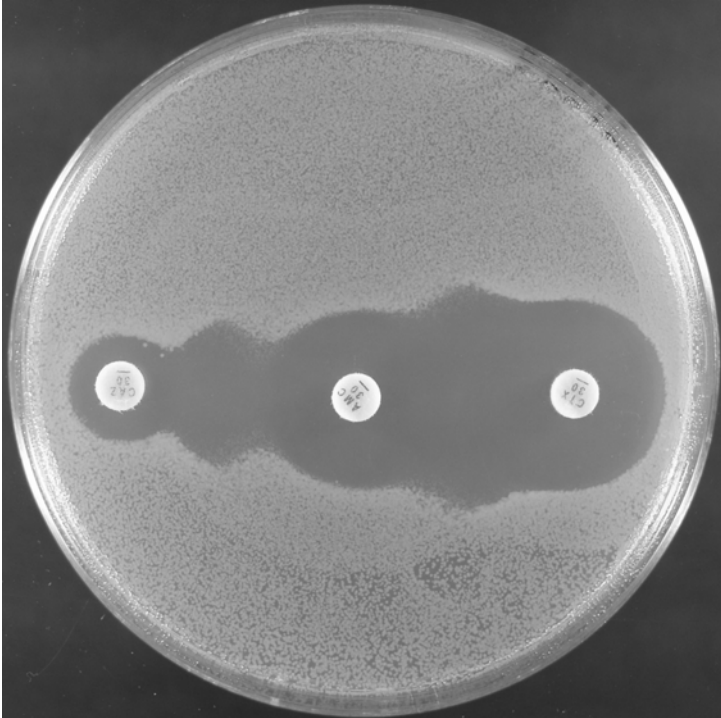


Figure 2

