Detection of Extended-Spectrum β-Lactamases in Klebsiellae with the Oxoid Combination Disk Method

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The Oxoid combination disk method for detecting extended-spectrum β-lactamases (ESBLs) depends on comparing the inhibition zones of cefpodoxime (10-µg) and cefpodoxime-plus-clavulanate (10- plus 1-µg) disks. The presence of clavulanate enlarged the zones for all of 180 ESBL-producing klebsiellae by ≥5 mm, whereas zones for cefpodoxime-susceptible isolates and cefpodoxime-resistant isolates with AmpC and K1 β-lactamases were enlarged by ≤1 mm. Good discrimination was achieved with either the NCCLS or the British disk method.

Extended-spectrum β-lactamases (ESBLs) are increasing sources of resistance to oxyimino-aminothiazolyl cephalosporins, especially in klebsiellae (1, 9). Most ESBLs are mutant TEM and SHV enzymes, but a few have different ancestries (4). Laboratory detection can be problematic, because some ESBLs do not confer obvious resistance to all their substrates in vitro and up to 35% of ESBL producers continue to be reported as susceptible to cefotaxime and ceftiraxone in Europe (1, 9). Accurate detection nevertheless is important because clinical failures arise even when the MICs of cephalosporins for ESBL producers are only 1 µg/ml (3, 13). Two detection strategies are in common use: (i) using ceftazidime or cefpodoxime as an indicator drug, and considering klebsiellae with reduced susceptibility to these drugs to be resistant to all oxyimino-aminothiazolyl cephalosporins (12) or (ii) screening for synergy between extended-spectrum cephalosporins and clavulanic acid. Synergy can be detected by double-disk tests (8), although the optimum separation of the disks is strain variable. Alternatively, commercial systems, such as Etests (5, 7) and Vitek (14), can be used. One obvious strategy is to compare the inhibition zones of cephalosporin disks with and without clavulanate added. This method has been used by several researchers (see, e.g., reference 10), and comparison of the zones given by cefotaxime (30-µg) and ceftazidime (30-µg) disks with or without clavulanate (10 µg) added is now advocated by the NCCLS (12). Such disks are available from several suppliers (Becton Dickinson, MAST, and Oxoid), and an evaluation was published recently (11). Cefpodoxime also seems an appropriate partner agent for clavulanate in such tests, since cefpodoxime itself is useful in ESBL screening tests. Accordingly, a cefpodoxime-based combination disk method has been developed (Oxoid, Basingstoke, Hampshire, United Kingdom) based on comparing the zones of cefpodoxime (10-µg) and cefpodoxime-plus-clavulanate (10- plus 1-µg) disks. We examined whether these disks reliably detected ESBL-positive klebsiellae and distinguished them from strains with AmpC enzymes and from Klebsiella oxytoca strains that hyperproduce chromosomal K1 (KOXY) β-lactamase.

Bacteria. The bacteria tested comprised Klebsiella pneumoniae and K. oxytoca collected from intensive-care unit (ICU) patients in multicenter surveys covering Western and Southern Europe in 1994 and in 1997 to 1998 (1, 9). One hundred eighty ESBL producers were included, selected on the basis of ceftazidime-ceftazidime-plus-clavulanate MIC ratios of ≥16 by agar dilution. The ESBLs produced by these isolates were partially identified previously by isoelectric focusing and PCR–single-strand conformation polymorphism for SHV types (15, 16), or by isoelectric focusing and PCR–restriction fragment length polymorphism for TEM types (16). Genes for a few of these ESBLs were sequenced (16). Molecular studies invariably detected an ESBL gene when the presence of an ESBL enzyme had been inferred from synergy between ceftazidime and clavulanate (15, 16). Also included were 19 K. oxytoca isolates that hyperproduced their K1 chromosomal β-lactamase, as demonstrated by antibiogram and kinetic studies (6), and 5 klebsiellae deduced to have AmpC β-lactamases on the basis of synergy between ceftazidime and the monobactam Ro 48-1256 (9). Both of the latter groups of isolates gave ceftazidime-ceftazidime-plus-clavulanate MIC ratios of ≤4 by agar dilution (9).

Disk test methods. The isolates were tested by the Oxoid combination disk method for ESBL Detection, which depends on comparing the inhibition zones given by cefpodoxime (CPD) (10-µg) and cefpodoxime-plus-clavulanate (CD01) (10- plus 1-µg) disks. Cefazidime (CAZ) (30-µg) disks (Oxoid) were tested in parallel as a control (8). The disk tests were performed (i) with confluent growth on Mueller-Hinton agar (Oxoid), in accordance with the NCCLS recommendations for nonfastidious bacteria (12), and (ii) with semiconfluent growth on Iso-Sensitest agar (Oxoid), in accordance with the recommendations of the British Society for Antimicrobial Chemotherapy (BSAC Standardized Disc Sensitivity Testing Method, Newsletter of the BSAC, Summer 1999). Zone diameters were measured to the nearest millimeter. A difference of ≥5 mm between the zones of the CD01 (10- plus 1-µg) and CPD (10-µg) disks was taken to indicate ESBL production, as advocated by the manufacturer. The zones of the CAZ (30-µg)
FIG. 1. Zone distributions by NCCLS methodology. (A) Distribution of inhibition zone differences (CD01 zone minus CPD zone) for ESBL producers (solid bars) and non-ESBL producers (open bars). (B and C) Zone diameters of CPD (10-μg) disks and CAZ (30-μg) disks, respectively, for ESBL producers (solid bars) and non-ESBL producers (open bars). Zone diameters indicating resistance, intermediate resistance, and possible ESBL positivity according to the NCCLS are situated to the left of the dashed, dotted-and-dashed, and solid vertical lines, respectively.
FIG. 2. Zone distributions by BSAC methodology. (A) Distribution of inhibition zone differences (CD01 zone minus CPD zone) for ESBL producers (solid bars) and non-ESBL producers (open bars). (B and C) Zone diameters of CPD (10-μg) disks and CAZ (30-μg) disks, respectively, for ESBL producers (solid bars) and non-ESBL producers (open bars). Zone diameters indicating resistance according to the BSAC are situated to the left of the solid vertical line.
and CPD (10-μg) disks additionally were reviewed against the susceptibility criteria of the NCCLS and BSAC, and against NCCLS criteria for predicting ESBL production (12).

**Inhibition zones for ESBL producers and susceptible controls.** The distributions of inhibition zone differences (the CD01 [10- plus 1-μg] zone minus the CPD [10-μg] zone) for the ESBL producers and ESBL-negative controls by NCCLS methodology are shown in Fig. 1A; those obtained by BSAC methods are shown in Fig. 2A. Regardless of which method was used, all 180 ESBL producers gave zones at least 5 mm larger with the CD01 (10- plus 1-μg) disks than with the CPD (10-μg) disks. The differences in zone diameters between the clavulanate-containing and non-clavulanate-containing disks were larger by use of the BSAC methodology (mean difference, 11.6 mm; standard deviation, 7.8 mm) than by use of the NCCLS method (mean difference, 10.0 mm; standard deviation, 4.2 mm). None of the 50 ESBL-negative control isolates gave a difference in zone diameter of more than 1 mm between the two disk types by either method.

The zones of CPD (10-μg) disks for ESBL producers and non-ESBL producers were better differentiated than those of CAZ (30-μg) disks, irrespective of whether NCCLS or BSAC methodology was used (compare Fig. 1B and C with Fig. 2B and C). This was so despite the fact that isolates had been categorized initially on the basis of synergy tests between cephalosporin, the AmpC producers were obviously resistant, and this resistance was not reversed by clavulanate, meaning that these strains were readily distinguishable from ESBL producers by the combination disk test. The behavior of the 19 K. oxytoca isolates that overexpressed the K1 enzyme. This discrimination is valuable for surveillance and for patient care, where it guides the choice of further antibiotics to test (cephalosporins and β-lactamase inhibitor combinations against ESBL producers, “fourth-generation” cephalosporins (céfpirone and céfepime) against AmpC producers, and ceftazidime against K1 hyperproducers). A limitation, irrespective of whether NCCLS or BSAC methods were followed, was that ca. 40% of K1 β-lactamase-hyperproducing K. oxytoca isolates appeared susceptible to the CPD (10-μg) disks. These strains would not have been confused with ESBL producers, but would have been categorized as cephalosporin susceptible unless tests were also performed with antibiotics that are better substrates for the K1 enzyme, notably aztreonam or cefuroxime.

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**REFERENCES**


