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Detection of Extended-Spectrum β-Lactamases in Members of the Family *Enterobacteriaceae*: Comparison of the Double-Disk and Three-Dimensional Tests

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The three-dimensional and clavulanate double-disk potentiation tests were compared as procedures for the detection of extended-spectrum β-lactamase production in 32 strains of *Escherichia coli* and *Klebsiella pneumoniae*, 31 of which produced TEM-1, TEM-2, TEM-3, TEM-4, TEM-5, TEM-7, TEM-8, TEM-9, TEM-10, TEM-12, TEM-101, SHV-1, SHV-2, SHV-3, SHV-4, SHV-5, CAZ-2, MIR-1, or an unidentified extended-spectrum β-lactamase with a pI of 5.95, with some strains producing multiple β-lactamases. The three-dimensional test, which was performed in conjunction with a routine disk diffusion test, detected extended-spectrum β-lactamase production in 26 of 28 (93%) of the strains that produced extended-spectrum β-lactamases. The clavulanate double-disk potentiation test detected extended-spectrum β-lactamases in only 22 of the 28 strains (79%) when it was performed as currently recommended. The three-dimensional test, when performed in conjunction with the disk diffusion test, offered the advantages of providing simultaneous information about both antibiotic susceptibility and extended-spectrum β-lactamase production, coupled with a greater sensitivity and earlier detection of extended-spectrum β-lactamases.

The recently recognized plasmid-mediated extended-spectrum β-lactamases (ESβs) of members of the family *Enterobacteriaceae* constitute an increasingly serious threat to current β-lactam therapy (11, 13). These enzymes can cause resistance to most penicillins, cephalosporins, and aztreonam (9, 19, 25). Some ESβs also hydrolyze cephamycins (2, 16). Strains that produce ESβs are often resistant to currently available β-lactamase inhibitor–β-lactam drug combinations as well (19, 24). The only β-lactam agents that consistently retain activity against ESβ-producing members of the family *Enterobacteriaceae* are carbapenems, penems, and temocillin (3, 6, 9, 10, 19, 21). Although currently of low incidence overall, ESβ-producing strains now occur worldwide and have been involved in epidemic spreads throughout institutions, particularly in Europe (4, 7, 9, 14, 19, 20). Institutional outbreaks appear to be a consequence of the ubiquity of members of the family *Enterobacteriaceae*, the selective pressure of heavy use of expanded-spectrum cephalosporins (22), and lapses in effective infection control measures (12, 18, 19, 22). Unfortunately, with most routine susceptibility tests, many of these strains do not appear to be resistant to β-lactams (2, 6, 12, 17–19). For example, in studies of more than 60 ESβ-producing strains, 29 to 75% of strains were susceptible to cefotaxime (12, 18) and 42.8% of strains were susceptible to ceftazidime (18) by disk diffusion testing. In another study of 46 ESβ-producing strains, the MIC of both cefotaxime and ceftazidime for 50% of strains tested was ≤16 μg/ml by agar dilution testing (6). Thus, failure to detect ESβ-producing members of the family *Enterobacteriaceae* can lead to delays in recognition that a problem exists until it has reached epidemic proportions.

Various approaches have been pursued to improve the laboratory detection of clinical isolates that produce ESβs. These include monitoring of susceptibility test results for slight decreases in susceptibility to expanded-spectrum cephalosporins or aztreonam (12, 18), the use of higher-than-standard inocula in MIC tests (21), and variations of the clavulanate double-disk potentiation procedure (12). All of these procedures have limitations. Slight decreases in susceptibility may not be recognized or may be due to resistance mechanisms other than ESβ production. Tests with high inocula lack interpretive criteria and can lead to false resistance. Double-disk tests can lack sensitivity because of problems of optimal disk spacing (24), the inability of clavulanate to inhibit all ESβs (2, 16, 26), and the inability of the test to detect ESβs in strains that also produce chromosomal cephalosporinases (5, 8).

Therefore, a study was designed to determine whether a modification of the three-dimensional test would improve detection of ESβs in isolates of the family *Enterobacteriaceae*. The three-dimensional test is a previously described modification of the disk diffusion test that generates data not only on antimicrobial susceptibility but also on the substrate profile of the β-lactamase(s) produced by the isolate being tested (1, 23). In this study, a modified form of the three-dimensional test was used to investigate the ability of the test to detect ESβ production by a panel of 32 clinical and laboratory strains of *Escherichia coli* and *Klebsiella pneumoniae*, 28 of which produced ESβs either alone or in combination with other β-lactamases. The double-disk test was performed simultaneously for comparative purposes.

MATERIALS AND METHODS

Strains. Tests were performed with 28 clinical and laboratory strains of *E. coli* and *K. pneumoniae* that produced the following ESβs: TEM-3, TEM-4, TEM-5, TEM-7, TEM-8, TEM-9, TEM-10, TEM-12, TEM-101, SHV-2, SHV-3, SHV-4, SHV-5, CAZ-2, MIR-1, TEM-1 and SHV-2, SHV-1 and SHV-3, MIR-1 and β-lactamases with pIs of 5.4 and 7.6, SHV-2 and β-lactamase with a pI of 6.5, TEM-1 and SHV-1 and β-lactamase with a pI of 5.95. Many of these strains were kindly provided by other investigators whose names

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TABLE 1. Routine disk diffusion tests of 28 ESβ-producing strains of members of the family *Enterobacteriaceae*

Susceptibility category	% Strains moderately susceptible or resistant to at least one antibiotic[a]											
	CTX	CRO	CAZ	ATM	CTX or CAZ	CTX or ATM	CTX, CAZ, or ATM	FOX	MA	CFP	PIP	IPM
Moderately susceptible	50	46	14	14	39	43	39	0	21	39	11	0
Resistant	18	18	36	29	43	32	43	7	43	18	89	0
Moderately susceptible or resistant	68	64	50	43	82	75	82	7	64	57	100	0

[a] The antibiotics tested were cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), aztreonam (ATM), cefoxitin (FOX), cefamandole (MA), cefoperazone (CFP), piperacillin (PIP), and imipenem (IPM). Percentages are rounded to the nearest whole number.

and laboratories are listed in the Acknowledgments. *E. coli* ATCC 25922 was used as a β-lactamase-negative control strain. *E. coli* ATCC 35218 (TEM-1) (15) and two additional strains of *E. coli* that produced TEM-2 and SHV-1 were used as non-ESβ, β-lactamase-positive controls.

Disk diffusion tests. Disk diffusion tests were performed and interpreted according to the recommendations of the National Committee for Clinical Laboratory Standards (15) by using BBL disks impregnated with piperacillin, aztreonam, imipenem, cefamandole, cefuroxime, cefoperazone, cefotaxime, ceftriaxone, ceftazidime, and cefoxitin. Disks were dispensed with a BBL Sensi-Disc 12-place dispenser. The direct three-dimensional test (see below) was performed on the same plate as the disk diffusion test.

Three-dimensional tests. The three-dimensional test is a modification of the disk diffusion procedure (15). It comprises an additional step which involves the application of a standardized bacterial inoculum into a circular slit in the agar 3 mm from the antibiotic disks, toward the interior of the plate (see Fig. 1). This modification provides the advantage of simultaneous determination of antibiotic susceptibility and β-lactamase substrate profile information (23). The latter is indicated by the presence or absence of characteristic distortions of the zone of inhibition. The three-dimensional inoculum is prepared at the same time as the inoculum for the disk diffusion test.

In this study, both inocula were obtained from an overnight agar culture of the test organism. The three-dimensional inoculum was prepared by suspending one loopful (10-μl loop) of the test organism in 0.5 ml of sterile tryptone soy broth (CM 129; Oxoid, Basingstoke, England). The suspension was vigorously mixed on a vortex mixer for 10 s and was then incubated at 35°C alongside the tube containing the inoculum for the disk diffusion test. Both tubes of inocula were incubated for the same period of time, i.e., until the disk diffusion test inoculum attained an optical density equal to that of a 0.5 McFarland standard. The three-dimensional inoculum produced by this procedure contained between 10^9 and 10^{10} CFU of cells that actively produced β-lactamase per ml.

In the direct three-dimensional test, after the surface of the susceptibility plate was inoculated by the method of the disk diffusion procedure (15), the agar was stabbed vertically with a sterile no. 11 scalpel blade so that the point of the blade passed to the bottom of the agar at a predetermined point 3 mm inside the position at which the antibiotic disks were to be placed. The blade was oriented perpendicular to the radius of the plate so that when the plate was rotated on a turntable, a circular slit was cut in the agar concentric with the margin of the plate. After completion of the circular cut, the blade was withdrawn and sterilized. The plate was then rotated again on the turntable and the three-dimensional inoculum was dispensed into the circular slit by using a 200-μl Pipetman pipet with a sterile pipet tip. The inoculum was dispensed so that the slit was filled but there was no overflow onto the agar surface. The moisture content of the agar was critical for this procedure. We strictly adhered to the recommendations of the National Committee for Clinical Laboratory Standards for refrigerated storage of plates in plastic and predrying before use (15). If the agar became too dry during storage, the three-dimensional slit might widen during inoculation, producing an undesired air gap in the agar. It was necessary for the sides of the slit to be in abutting contact for the test to work. After the completion of the three-dimensional inoculation, the BBL Sensi-Disc dispenser was used to place the disks on the agar 3 mm outside of the inoculated circular slit, and the plate was incubated in the usual manner.

Conventional (two-dimensional) disk diffusion susceptibility test results were measured according to the recommendations of the National Committee for Clinical Laboratory Standards (15). If the zones were distorted, accurate diameters could be calculated by doubling the measurements of radii at undistorted parts of the zones, as shown in previous studies (23). Both susceptibility results and three-dimensional results were determined from the same plate unless the indirect form of the test was used (see below).

Enzymatic inactivation of the test antibiotic was detected by inspection of the margin of the inhibition zone in the vicinity of its intersection with the circular three-dimensional inoculation. Inactivation of the drug as it diffused through the inoculated slit resulted in a distortion or discontinuity in the usually circular inhibition zone or the production of discrete colonies in the vicinity of the inoculated slit (see Fig. 1 to 3). Although the evidence of drug inactivation was sometimes only a subtle distortion, the test result was still recorded as positive for enzymatic drug inactivation, i.e., the test was assessed qualitatively, not quantitatively (see Fig. 2). The absence of any distortion of the zone margin or discrete colony formation in the vicinity of the three-dimensional inoculation indicated that there was no detectable drug inactivation.

When inhibition zones were small or absent, the β-lactamases of resistant strains were investigated by the indirect three-dimensional test. The indirect test was performed by inoculating the surface of the agar with a fully susceptible assay strain such as *E. coli* ATCC 25922 and then inoculating the circular cut in the agar with the suspension of the test organism. Although the indirect test precluded the simultaneous determination of antibiotic susceptibilities, it permitted investigation of the β-lactamases of organisms for which inhibition zones were too small to yield three-dimensional results when the test was performed in the previously described manner.

Double-disk test. The ESβ-producing strains were also tested by the clavulanate double-disk potentiation procedure of Jarlier et al. (12). In this test, a plate was inoculated as described above for a standard disk diffusion test. Disks containing aztreonam and expanded-spectrum cephalosporins were then placed 30 mm (center to center) from an amoxicillin-clavulanate disk prior to incubation. After overnight incubation at 35°C, the production of an ESβ by the test organism was inferred by the presence of characteristic distortions of the inhibition zones indicative of clavulanate potentiation of the activity of the test drug. Negative double-disk tests were repeated with a disk spacing of 20 mm (center to center).

RESULTS

Disk diffusion antibiotic susceptibility tests. The results of standard disk diffusion tests were analyzed to determine whether a pattern of reduced susceptibility that was predictive of the presence of ESβs could be identified. Eighty-two percent of the ESβ-producing strains were found to have reduced susceptibilities (zones indicative of resistance or moderate susceptibility) to expanded-spectrum cephalosporins and/or aztreonam if results from tests with both cefotaxime and ceftazidime were grouped together. Only 43 to 68% of strains exhibited reduced susceptibilities to single drugs from this group (Table 1). In tests with either a cefotaxime or a ceftriaxone disk, reduced susceptibility was not detected in some strains that produced TEM-5, TEM-7, TEM-12, TEM-101, SHV-3, or CAZ-2. In tests with either ceftazidime or aztreonam, reduced susceptibility was not detected in some strains that produced TEM-3, TEM-4, TEM-5, TEM-7, TEM-12, TEM-101, SHV-2, SHV-3, or the enzyme with a pI of 5.95. The reduced susceptibility associated with CAZ-2 was not detected in tests with aztreonam. When results for cefotaxime and ceftazidime were examined together, reduced susceptibility was not detected in any strains that produced TEM-7, TEM-12, TEM-101, or SHV-3.

Three-dimensional tests. The three-dimensional tests with cefotaxime and ceftriaxone were the most sensitive indicators of ESβ production. All ESβs except TEM-12 were detected in three-dimensional tests with cefotaxime or ceftriaxone (Table 2). Only one ESβ (MIR-1) was detected in three-dimensional tests with cefoxitin, and no ESβs were detected in three-dimensional tests with ceftazidime or aztreonam. Although three-dimensional tests with the other β-lactam antibiotics examined indicated the presence of β-lactamase activity, these antibiotics were not useful for the detection of ESβs, because they were also hydrolyzed by non-ESβs such as TEM-1, TEM-2, and SHV-1 (Table 2 and Fig. 1 to 3). No evidence of β-lactamase activity was observed in three-dimensional tests with the β-lactamase-negative control strain *E. coli* ATCC 25922. The non-ESβ controls, TEM-1, TEM-2, and SHV-1, did not give positive three-dimensional tests with cefotaxime, ceftriaxone, ceftazidime, or aztreonam.

Of the 28 ESβ-producing strains examined in this study, 20 were examined for ESβs by the direct three-dimensional test, and 8 had to be examined by the indirect three-dimensional test. ESβs were detected in 18 of 20 strains by direct three-dimensional tests and 8 of 8 strains were detected by indirect three-dimensional tests. The two strains in which ESβs were not detected produced the enzyme TEM-12.

Double-disk tests. ESβ production was detected in double-

TABLE 2. Three-dimensional test results[a]

Antibiotic	Non-ESβ-producing controls			ESβ producers															
No enzyme[b]	TEM-1	TEM-2	SHV-1	TEM-3	TEM-4	TEM-5	TEM-7	TEM-8	TEM-9	TEM-10	TEM-12	TEM-101	SHV-2	SHV-3	SHV-4	SHV-5	CAZ-2	MIR-1	Enzyme with pI of 5.95
Cefotaxime	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ceftriaxone	−	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Ceftazidime	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aztreonam	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cefoxitin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cefoperazone	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cefamandole	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Piperacillin	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] +, evidence of antibiotic inactivation in the three-dimensional test; −, no evidence of drug inactivation in the three-dimensional test.
[b] *E. coli* ATCC 25922.

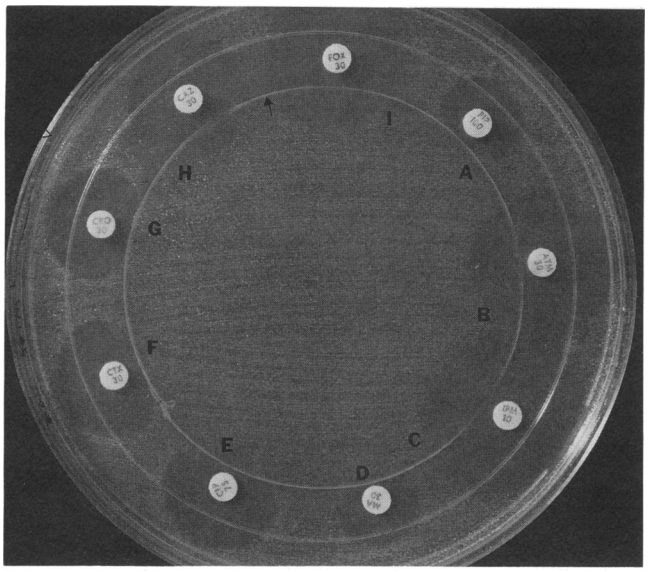

FIG. 1. Direct three-dimensional test of SHV-2-producing *E. coli* MISC 208. The circular three-dimensional inoculation (indicated by the arrow) intersected the inhibition zone margins to produce major distortions (i.e., positive three-dimensional test results) that indicated enzymatic inactivation of piperacillin (A), cefamandole (D), cefoperazone (E), cefotaxime (F), and ceftriaxone (G). Distortions did not occur (i.e., negative three-dimensional test results) in tests with aztreonam (B), imipenem (C), ceftazidime (H), or cefoxitin (I). The outer circle is the plastic rim on the bottom of the petri dish.

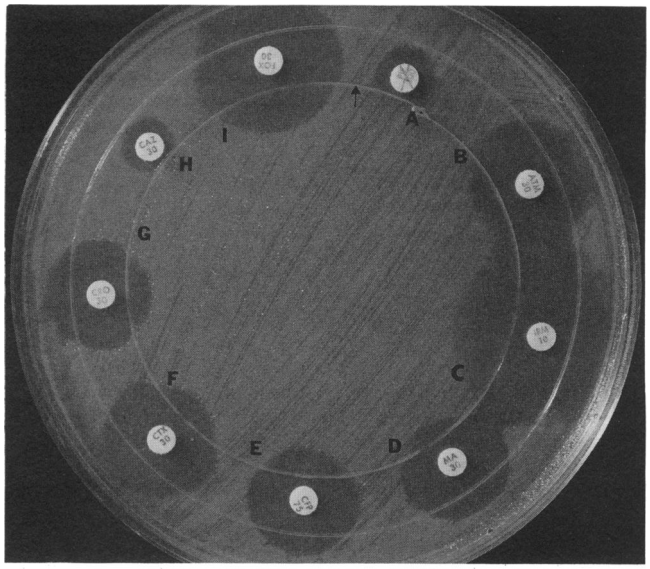

FIG. 2. Direct three-dimensional test of CAZ-2-producing *E. coli* MISC 234. The three-dimensional inoculation (arrow) resulted in minor distortions that indicated antibiotic inactivation (i.e., positive three-dimensional test results) at the intersection with the zone margins of cefamandole (D), cefoperazone (E), cefotaxime (F), and ceftriaxone (G). Distortions did not occur in tests with aztreonam (B), imipenem (C), or cefoxitin (I). The inhibition zones were too small to interpret in the direct three-dimensional test in tests with piperacillin (A) and ceftazidime (H).

disk tests with 22 (79%) of the 28 ESβ-producing strains when they were tested at the recommended disk spacing of 30 mm. When tests with the six strains that were not detected were repeated by using the closer disk spacing of 20 mm, one additional strain exhibited a distorted zone margin suggestive of ESβ production. That is, 23 (82%) of the 28 strains were detected by the double-disk method by using two disk spacings. Of the six ESβ-producing strains that were not detected with the recommended disk spacing of 30 mm, four strains produced multiple β-lactamases and two produced the clavulanate-insensitive MIR-1 enzyme (Table 3). All ESβ-negative control strains gave negative double-disk test results.

DISCUSSION

In this study, routine disk diffusion tests yielded moderately susceptible or resistant results in 43 to 68% of tests in which one of the four drugs cefotaxime, ceftriaxone, ceftazidime, or aztreonam was tested against ESβ-producing organisms. If both cefotaxime and ceftazidime were tested, the index of suspicion increased to only 82%. Thus, it is clear that additional specific tests for the detection of ESβs are needed.

Of the two specific tests investigated in this study, the three-dimensional test provided a more sensitive procedure for the detection of ESβ-producing strains than did the double-disk test. Twenty-six of the 28 study strains (93%) were presumptively identified as ESβ-producers from three-dimensional tests with cefotaxime or ceftriaxone, while only 22 of the 28 ESβ-producing strains were detected by the recommended form of the double-disk test. One additional ESβ-producing strain was detected when closer spacing between disks was used.

The detection of 18 (64%) of the ESβ-producing strains in the direct three-dimensional test suggested that a laboratory that uses disk diffusion tests in combination with the three-dimensional test would be able to confirm ESβ production in many strains from the initial disk susceptibility test. This is 1 day sooner than is currently possible when the special tests used to detect ESβs, like the double-disk test, are initiated only after interpretation of the susceptibility test result. The strains that cannot be confirmed as ESβ producers from the direct test because of their resistance to cefotaxime or ceftriaxone should be automatically regarded with suspicion and followed up with the indirect test.

Ideally, clinical laboratories should be able to detect new and clinically important antibiotic resistance mechanisms such as ESβs in time to contribute to clinical and infection control decision making. The continuing worldwide trans-

TABLE 3. Strains that yielded negative double-disk test results[a]

Organism	β-Lactamase	
	ESβ	Other
K. pneumoniae JW7	Enzyme with pI of 5.95[b]	TEM-1, SHV-1
K. pneumoniae JW8	SHV-2	Enzyme with pI of 6.5
K. pneumoniae JW1	SHV-2	TEM-1
K. pneumoniae MISC 304	MIR-1	Enzymes with pIs of 5.4 and 7.6
E. coli MISC 305	MIR-1	None
E. coli MG4	TEM-12	TEM-1

[a] Clavulanate double-disk potentiation test of Jarlier et al. (12) with disk spacing of 30 mm (center to center).
[b] Unidentified ESβ of pI 5.95.

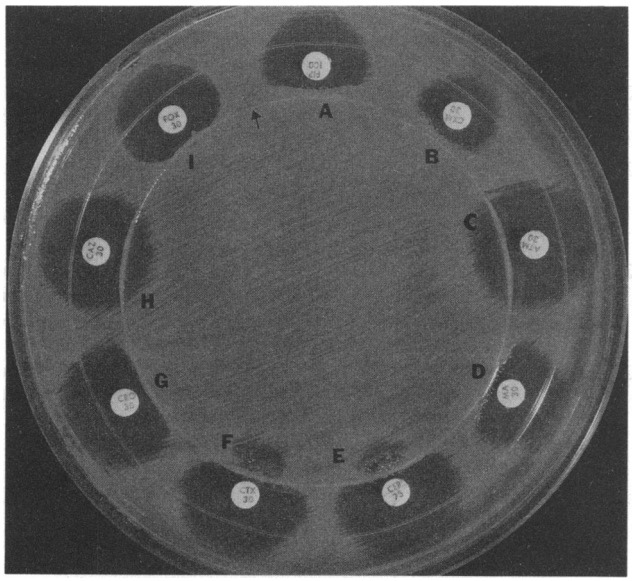

FIG. 3. Indirect three-dimensional test to investigate *K. pneumoniae* MISC 304 that produced three β-lactamases (MIR-1 and enzymes with pIs of 5.4 and 7.6). In this test, *K. pneumoniae* MISC 304 was inoculated only into the circular three-dimensional slit inoculation (arrow). The fully susceptible strain *E. coli* ATCC 25922 was used as the surface lawn culture to assay for the β-lactamase activity associated with *K. pneumoniae* MISC 304. Major zone distortions that indicated antibiotic inactivation (i.e., positive three-dimensional test results) occurred in tests with piperacillin (A), cefuroxime (B), cefamandole (D), cefoperazone (E), cefotaxime (F), ceftriaxone (G), and cefoxitin (I) but not in tests with aztreonam (C) or ceftazidime (H). Note the growth of small colonies in the cefoperazone (E) and cefotaxime (F) zones in the vicinity of the three-dimensional inoculation site. The small colonies in this part of the zone also indicated enzymatic drug inactivation (i.e., positive three-dimensional test results).

mission of ESβs is partly a result of the fact that clinical laboratories are not able to detect these enzymes as effectively or rapidly as is needed. In this study, a collection of known ESβ-producing strains was studied. To properly assess the potential of the three-dimensional test, it is desirable that the technique also be evaluated by field testing with routine clinical isolates. If its utility is confirmed, the three-dimensional test may help clinical laboratories to provide a faster and more sensitive means of detecting ESβs, and it may also be used to generate information about other β-lactamases.

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