Evaluation of Four Commercially Available Extended-Spectrum Beta-Lactamase Phenotypic Confirmation Tests

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Extended-spectrum beta-lactamase (ESBL) production in members of the Enterobacteriaceae can confer resistance to extended-spectrum cephalosporins, aztreonam, and penicillin. As such, the accurate detection of ESBL producers is essential for the appropriate selection of antibiotic therapy. Twenty previously characterized isolates and 49 clinical isolates suspected of ESBL production were tested by four ESBL phenotypic confirmatory methods for accuracy and ease of use. The four ESBL phenotypic confirmation tests included Dried MicroScan ESBL plus ESBL Confirmation panels (Dade Behring, Inc., West Sacramento, Calif.), Etest ESBL (AB BIODISK, Piscataway, N.J.), Vitek GNS-120 (bioMérieux, Inc., Hazelwood, Mo.), and BD BBL Sensi-Disk ESBL Confirmatory Test disks (BD Biosciences, Sparks, Md.). Results were compared to frozen microdilution panels prepared according to NCCLS specifications, and discrepant isolates were sent for molecular testing. The test sensitivities for the ESBL phenotypic confirmatory test methods used in this study were as follows: MicroScan ESBL plus ESBL confirmation panel, 100%; VITEK 1 GNS-120, 99%; Etest ESBL, 97%; and BD BBL Sensi-Disk ESBL Confirmatory Test disks, 96%. The test specificities were as follows: BD BBL Sensi-Disk ESBL Confirmatory Test disks, 100%; MicroScan ESBL plus ESBL confirmation panel and VITEK 1 GNS-120, 98%; and Etest ESBL, 94%. All methods were easy to perform; however, the Etest method required more expertise to interpret the results. All tests offer a feasible solution for confirming ESBL production in the clinical laboratory.

Klebsiella pneumoniae and Escherichia coli have been recognized to contain group 2 beta-lactamas (6). The pharmaceutical industry developed novel classes of beta-lactam drugs to protect the beta-lactam ring from hydrolysis by these enzymes. One of the beta-lactam drug classes resulted from the addition of an oxyimino side chain to a 2-amino-5-thiazolyl nucleus cephalosporin base antibiotic, which resulted in antibiotics resistant to hydrolysis by the TEM-1 or -2 and SHV beta-lactamases. These oxyimino-cephalosporin (ceftriaxone, cefotaxime [CTX], and ceftazidime [CAZ]) antibiotics have an extended spectrum of activity compared to the narrow- and broad-spectrum cephalosporins. Shortly after the introduction of these extended-spectrum beta-lactam drugs, gram-negative bacilli were detected that contained mutated versions of the SHV and TEM beta-lactamases and demonstrated resistance to these extended-spectrum antibiotics (22). In 1983, the first reported extended-spectrum beta-lactamase (ESBL) producer occurred in K. pneumoniae (15) followed by the detection of an ESBL producer in E. coli in 1987 (1). So named for their ability to hydrolyze extended-spectrum cephalosporins, these plasmid-borne beta-lactamases are mostly descendants of the TEM and SHV enzymes (10, 19). To date, there are over 180 different ESBL enzymes that cause resistance to penicillins, cephalosporins, and aztreonam (http://www.lahey.org/studies/webt.htm). Originally observed only in E. coli and Klebsiella spp., ESBL production has begun to occur within other gram-negative bacilli (5, 11, 16, 18, 21). These ESBL-producing organisms have been associated with health care-associated outbreaks (2, 4, 9, 11, 12, 18).

The clinical microbiology laboratory has the task of screening and confirming isolates for ESBL production. This is a challenge for the laboratory to detect ESBL-containing gram-negative bacilli because they can appear susceptible in vitro to certain beta-lactam antimicrobial agents yet result in clinical treatment failure (19, 25). Currently the NCCLS documents recommend screening of ESBL production in E. coli, K. pneumoniae, and Klebsiella oxytoca by using the antimicrobial agents cefpodoxime, CAZ, aztreonam, CTX, and ceftriaxone. The use of several antimicrobial agents increases the sensitivity of ESBL detection (17). Confirmatory testing should be performed on organisms in which the ESBL screen may indicate ESBL production. Phenotypic confirmatory testing involves testing the E. coli, K. pneumoniae, or K. oxytoca isolates against CAZ and CTX alone and in combination with clavulanic acid (CTX/CA and CAZ/CA, respectively). NCCLS has recommended that susceptible penicillin, cephalosporin, and aztreonam results in confirmed ESBL producers be reported as resistant (17). Confirmatory tests for ESBL production that are commercially available in the United States were compared for sensitivity and specificity. In this study, 32 previously defined challenge isolates and 90 clinical isolates suspicious for ESBL production were tested by each of the above methods and compared to an NCCLS-approved frozen broth microdilution susceptibility test for the phenotypic confirmation of ESBL production.

(This study was presented previously [Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. A-055 and C-262, 2003].)
the presence of a phantom zone, or deformity, of the CTX or CAZ strip. A
CAZ at one end and CTX or CAZ with CA at the other end were performed in
to detect ESBL production. An ESBL producer had a
with BD BBL CTX and CAZ disks alone and in combination with CA was used
for ESBL production were used as clinical isolates in this study.

Materials and Methods

Study design. Four commercially available methods to confirm ESBL produc-
tion were evaluated and compared for ease of use. The methods tested were
MicroScan ESBL plus ESBL confirmation panels (Dade Behring Inc., West
Sacramento, Calif.), Etest ESBL (AB BIODISK, Piscataway, N.J.), VITEK 1
(bioMérieux, Inc., Hazelwood, Mo.), and BD BBL Sensi-Disk ESBL. Confirma-
tory Test disks (BD Biosciences, Sparks, Md.). Frozen broth microdilution ESBL
panels prepared according to NCCLS specifications were used for comparison.
Thirty-two previously characterized challenge isolates, including positive and
negative strains whose characterization was not known at the time of study, and
90 clinical isolates were tested. Isolates showing discrepancies by phenotypic
methods were further characterized by isoelectric focusing and PCR analysis at
Creighton University as previously described (13, 20, 23, 24).

Challenge and clinical isolates. The challenge isolates include 24 E. coli
isolates (13 ESBL producers and 11 non-ESBL producers) and 8 K. pneumoniae
isolates (all ESBL producers). The challenge strains, identified only by code
numbers, were subcultured once before the confirmatory testing was performed.

Thirty-eight E. coli isolates, 50 K. pneumoniae isolates, and 2 K. oxytoca isolates
(n = 90) from two hospitals that were screen positive by NCCLS criteria for
ESBL were determined by a
3 twofold-concentration decrease in MICs of either CAZ or CTX in the
presence of a fixed concentration of CA versus the MIC when tested alone.

BD BBL Sensi-Disk ESBL Confirmatory Test disks. The disk diffusion method
with BD BBL CTX and CAZ disks alone and in combination with CA was used to
detect ESBL production. An ESBL producer had a ≥5-mm-zone size differ-
ce between the CTX/CA or CAZ/CA disks compared to disks without the CA.

Etest ESBL confirmation. Etest strips with gradient concentrations of CTX or CAZ at one end and CTX or CAZ with CA at the other end were performed in
accordance with the guidelines of the manufacturer for ESBL production. ESBL
production was determined by a ≥3 twofold-concentration decrease in the MIC
of CTX or CAZ in the presence of CA. ESBL production was also identified by
the presence of a phantom zone, or deformity, of the CTX or CAZ strip. A
nondeterminable (ND) result was declared when the MICs were greater than the
range of MICs of the respective Etest ESBL test strip.

VITEK 1 ESBL confirmation. The VITEK 1 automated system using the
GNS-120 card was performed in accordance with the guidelines of the manu-
facturer for ESBL detection. Software ≥VTK R07 was needed in order to in-
terpret the reduction in growth due to CTX/CA or CTZ/CA compared to either
CTX or CAZ alone. Results were reported as ESBL positive or ESBL negative.

Frozen broth microdilution MIC panels for ESBL confirmation. An NCCLS-
approved broth microdilution MIC frozen panel for ESBL confirmation was used
as the NCCLS reference method. The reference panels were made in accordance
with NCCLS guidelines and were stored at −70°C before use. Panels were read
manually following 16 to 20 h of incubation. Confirmatory testing uses both CTX
and CAZ, alone and in combination with K CA. ESBL production was deter-
mined by a ≥3 twofold-concentration decrease in MICs of either CAZ or CTX
in the presence of a fixed concentration of K CA versus its MIC when tested alone.

Results

MicroScan ESBL results. The MicroScan ESBL plus ESBL confirmation test method detected ESBL production in 21 of 32 challenge isolates (Table 1). The MicroScan results for the challenge strains were in complete agreement with previously determined enzyme characteristics. ESBL production was detected in 62 of 90 of the clinical isolates, one of which showed false ESBL production (Table 1). Clinical isolate L54, identi-
fied as K. oxytoca, was confirmed as an ESBL producer by the MicroScan ESBL plus ESBL confirmation test method. The discrepant results, which indicated ESBL production, were a CTX MIC of 4 μg/ml and a MIC of <0.12/4 for CTX/CA. This discrepant isolate was later characterized as an OXY-2 K1 that
was inhibited by CA (Table 2). The overall sensitivity and

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolate identification</th>
<th>Enzyme characterization</th>
<th>Result by:*</th>
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<tbody>
<tr>
<td>Challenge</td>
<td></td>
<td></td>
<td>MicroScan</td>
</tr>
<tr>
<td>39661</td>
<td>E. coli</td>
<td>ESBL, SHV-4-like</td>
<td>−</td>
</tr>
<tr>
<td>39664</td>
<td>K. pneumoniae</td>
<td>ESBL, TEM-10</td>
<td>+</td>
</tr>
<tr>
<td>41500</td>
<td>E. coli</td>
<td>ESBL, TEM</td>
<td>+</td>
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<tr>
<td>41510</td>
<td>E. coli</td>
<td>ESBL, TEM-like</td>
<td>+</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td>MicroScan</td>
</tr>
<tr>
<td>L54</td>
<td>K. oxytoca</td>
<td>No ESBL, OXY-2 K1</td>
<td>+</td>
</tr>
<tr>
<td>L68</td>
<td>E. coli</td>
<td>ESBL, SHV-5-like and AmpC</td>
<td>+</td>
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</table>

a +, positive; −, negative.
specificity by the MicroScan test method were 100 and 98%, respectively.

**BD BBL Sensi-Disk results.** Using the BD BBL disk method, 19 of 32 challenge isolates were shown to have ESBL production (Table 1). Two challenge strains that tested negative for ESBL production by the BD BBL disk test method had previously been characterized as having a TEM-like enzyme. Zone sizes for challenge isolate 41500 of 24 mm for CTX and 28 mm for CTX/CA and 24 mm for CAZ and 27 mm for CAZ/CA did not confirm this isolate as an ESBL producer by the BD BBL disk method. Likewise, challenge isolate 41510 was not confirmed as an ESBL producer. Zone sizes for that isolate were 21 mm for CTX and 25 mm for CTX/CA and 21 mm for CAZ and 24 mm for CAZ/CA. The BD BBL disk method detected 61 of the ESBL producers of the clinical isolates (Table 1). There was one discrepant clinical isolate (*E. coli* L68) that was subsequently determined to contain a SHV-5-like enzyme along with an AmpC enzyme (Table 2). Isolate L68 had zone sizes of 15 and 19 mm for CTX and CTX/CA, respectively, and 8 and 10 mm for CAZ and CAZ/CA, respectively. The overall sensitivity and specificity by the BD BBL disk test method were 96 and 100%, respectively.

**Etest ESBL results.** The Etest ESBL confirmation test method detected ESBL production in 18 of 32 challenge isolates (Table 1). The MICs for three of the challenge isolates were greater than the range used in the gradient strip and were reported as ND. Two challenge isolates, 39661 and 39664, did not show ESBL production by the Etest method, but a SHV-4-like enzyme and TEM-10 enzyme were present (Table 2). The MICs for clinical isolate L54 were 0.094 g/ml for CTX and 0.09 g/ml for CAZ and 0.12 g/ml for CAZ/CA. Challenge isolate 39664 also was not confirmed as an ESBL producer by this method. The MIC results for challenge isolate 39664 were ≤0.25 µg/ml for CTX and 0.09 µg/ml for CTX/CA and <0.5 µg/ml for CAZ and 0.12 µg/ml for CAZ/CA. Challenge isolate 39664 also was not confirmed as an ESBL producer by this method. The MIC results for challenge isolate 39664 were ≤0.25 and 0.125 µg/ml for CTX and CTX/CA, respectively; and <0.5 and 0.38 µg/ml for CAZ and CAZ/CA, respectively. The results from the clinical isolates were as follows: 59 of 90 showed ESBL production and 21 of 90 were ND (Table 1). A 100% sensitivity was observed, since all 58 true-positive clinical isolates that could be measured were detected. The specificity was 91%, with one false-positive result. Clinical isolate L54 (*K. oxytoca*), which showed false ESBL production, was determined to have an OXY-2 K1 enzyme (Table 2). The MICs for clinical isolate L54 were 2 and 0.094 µg/ml for CTX and CTX/CA, respectively, and <0.5 and 0.19 µg/ml for CAZ and CAZ/CA, respectively. Overall sensitivity and specificity by the Etest test method were 97 and 94%, respectively.

**VITEK 1 ESBL results.** The VITEK 1 ESBL test method correctly identified 21 of the 32 challenge isolates for ESBL production. The VITEK 1 test detected 59 of 90 ESBL producers of the clinical isolates. Discrepancies by this test method included both a false-positive result and a false-negative result. Clinical isolate L54 (*K. oxytoca*) was determined to have an OXY-2 K1 enzyme. The other discrepant isolate (clinical isolate L68), identified as *E. coli*, was shown to contain both SHV-5 and AmpC enzymes (Table 2). The overall sensitivity and specificity by the VITEK 1 test method were 99 and 98%, respectively.

**Frozen broth microdilution MIC results.** The frozen broth microdilution MIC panel detected all 21 of 32 challenge isolates with ESBL production correctly; however, the frozen MIC panel incorrectly detected ESBL production in challenge strain L54 (Tables 1 and 2). The MICs of CTX (2 µg/ml) and CTX/CA (0.12/4 µg/ml) indicate that challenge isolate L54 was an ESBL producer by the frozen broth microdilution reference method. Challenge strain L54 (*K. oxytoca*) was shown to contain an OXY-2 K1 enzyme. The overall sensitivity and specificity by the frozen microdilution MIC method were 100 and 98%, respectively.

**DISCUSSION**

The phenotypic confirmation of ESBL production is recommended by NCCLS (17). Isolates which are screen positive for ESBL production should be confirmed by testing with CTX and CAZ alone and in combination with CA. Both CTX and CAZ with and without CA are tested to ensure detection of ESBL production. Although CAZ currently detects most ESBLs in the United States, the use of only one drug combination will not detect all ESBL producers, as can be seen in Table 3. Furthermore, ESBLs of the CTX-M group are increasing and spreading throughout the world. These enzymes are more active against CTX than against CAZ (3).

Four commercially available methods and a reference method to confirm the presence of ESBL production were compared in this study. The test sensitivities for the ESBL confirmatory test methods were as follows: the reference microbroth and MicroScan ESBL plus ESBL confirmation panel, 100%; VITEK 1 GNS-120, 99%; Etest ESBL, 97%; and BD BBL Sensi-Disk ESBL Confirmatory Test disks, 96%. The test specificities for the ESBL confirmatory test methods were as follows: BD BBL Sensi-Disk ESBL Confirmatory Test disks, 96%. The test specificities for the ESBL confirmatory test methods were as follows: BD BBL Sensi-Disk ESBL Confirmatory Test disks, 100%; the reference broth microdilution, MicroScan ESBL plus ESBL confirmation panel and VITEK 1 GNS-120, 98%; and Etest ESBL, 95%. Using the Etest ESBL confirmatory test, 24 isolates had ND ESBL production because MIC results were higher than the MIC test range.

Discrepancies between methods were noted in four challenge isolates and two clinical isolates. Challenge isolate 39661 (*E. coli*) was shown to have ESBL production by the reference broth microdilution and MicroScan methods, but it did not show ESBL production with the BD BBL disks or Etest.
or VITEK methods. The challenge isolate was determined to have an SHV-4 like enzyme and thus was classified as an ESBL producer. Challenge isolate 39664 (K. pneumoniae) tested positive for ESBL production by the reference method, MicroScan, BD BBL disks, and VITEK, but did not show ESBL production by the Etest method. This isolate was determined to be an ESBL producer with a TEM-10 enzyme. Challenge isolates 41500 and 41510 (both E. coli) failed to show ESBL production by the BD BBL disk method but did show ESBL production by all other methods. Both challenge isolates were determined to be ESBL producers as challenge isolate 41500 was determined to have a TEM enzyme and challenge isolate 41510 was shown to have a TEM-like enzyme. Clinical isolate L54 (K. oxytoca) was problematic for most test methods; only the BD BBL disk method had the highest specificity.

A more recently recognized mechanism of extended-spectrum beta-lactam resistance results from mutated ESBL enzymes which are resistant to inactivation by CA. Amino acid substitutions at key locations, such as glycine replacing serine at site 130, make the ESBL refractory to the inhibitory activity of CA (7). These inhibitor-resistant ESBLs are classified as 2hr in the Bush-Jacoby-Medeiros classification system (6). The inhibitor-resistant ESBLs are phenotypically similar to ampC hyperproducers. The phenotypic indicator which suggests you might have an inhibitor-resistant ESBL is the susceptibility of the isolate to cephapemycins (cefoxitin or cefotetan). The ampC beta-lactamases cause resistance to cephapemycins. The two most likely explanations for a negative confirmatory test for an ESBL enzyme are (i) an inhibitor-resistant ESBL or (ii) failure of the confirmatory test, as described in this report. Such isolates should be tested in a different confirmatory test or submitted to a reference laboratory for molecular characterization of the beta-lactam resistance mechanism.

All test methods were easy to perform and interpret. MicroScan had easy-to-read end-points, but their panels must be incubated and read manually. Twenty-four isolates gave the result ND by the Etest method, because the MICs for them were higher than the MICs available on the test strip, which would need further testing to confirm ESBL production. The BD BBL disk test was both easy to perform and interpret. The GNS-120 card method is read with the VITEK automated system and is reported as positive or negative for ESBL production. The MicroScan method had the highest sensitivity and the BD BBL method had the highest specificity.

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