Rapid Detection of ESBL-Producing Enterobacteriaceae in Blood Cultures

Laurent Dortet, Laurent Poirel, Patrice Nordmann

We rapidly identified extended-spectrum β-lactamase (ESBL) producers prospectively among 245 gram-negative bacilli–positive cultured blood specimens using the Rapid ESBL Nordmann/Dortet/Poirel test and direct bacterial identification using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This combination identified ESBL-producing Enterobacteriaceae within 30 min and had high predictive values.

An essential parameter for improving the outcome of sepsis is early implementation of appropriate antibiotic therapy (1–5). Recently, using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) technology directly with blood cultures was found to help guide clinical management of bacteremia caused by gram-negative bacteria (GNB) (6). Resistance to broad-spectrum cephalosporins is spreading rapidly among Enterobacteriaceae, mostly related to acquisition of extended-spectrum β-lactamases (ESBLs) (7). ESBL-producing Enterobacteriaceae (ESBL-E) are usually resistant to most β-lactams except cefamycins and carbapenems.

Using PCR-based molecular techniques on positive blood cultures has been proposed for rapid identification of ESBLs (8); however, trained personnel and expensive material are required for their use. In addition, for the TEM- and SHV-type enzymes, detailed gene sequence analysis is required for differentiating narrow-spectrum β-lactamases from ESBLs.

Rapid identification of ESBL producers is possible by using the ESBL Nordmann/Dortet/Poirel (NDP) test (9), which is based on the biochemical detection of the hydrolysis of the b-lactam ring of cefotaxime (a broad-spectrum cephalosporin). Presence of these bacteria has previously been evaluated with cultured bacteria and with spiked blood cultures (9).

In this study, we evaluated the ESBL NDP test prospectively in clinical settings directly from blood cultures. Identification of the bacterial species was done concomitantly from blood cultures by using enhanced MALDI-TOF procedures.

The Study
During November 2012–May 2013, we studied a single blood culture positive for GNB from each of 245 patients hospitalized at the Bicêtre hospital, a 950-bed hospital located in a suburb of Paris. Positivity of blood cultures was detected by using the BacT/Alert system (bioMérieux, La Balme-les-Grottes, France). After obtaining Gram stain results, we tested the blood cultures positive for GNB directly for 1) ESBL-E by using the ESBL NDP test, and 2) species identification by using the MALDI-TOF MS technique.

We adapted the protocol of the ESBL NDP test for detection of the ESBL-E from blood cultures (9) (Online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/3/14-1277-Techapp1.pdf). The detailed MALDI-TOF MS protocol using the VITEK MS system (bioMérieux) is described in Detailed Methods in the Technical Appendix.

We performed antibiotic susceptibility testing (AST) by the disk diffusion method using bacterial colonies grown from blood cultures according to the Clinical Laboratory Standards Institute (CLSI) recommendations (10). The same MALDI-TOF technology and the API Gram negative Identification product (bioMérieux) were used for confirmatory identification of bacteria. AST results, obtained 48 h after blood cultures were identified as positive, were interpreted according to the CLSI breakpoints, as updated in 2014 (10). MIC of cefotaxime, ceftazidime, and cefepime were determined on Muller-Hinton (MH) agar and MH agar supplemented with 4 µg/mL of tazobactam (final concentration).

We used the double-disk synergy test (DDST) for the phenotypic detection of ESBL producers (11), according to the CLSI recommendations. For each sample, 1 disk contained cefotaxime, ceftazidime, or cefepime, and a second disk contained ticarcillin and clavulanate (10). The DDST was also performed on MH agar plates (bioMérieux) containing cloxacillin (150 mg/L) to inhibit cephalosporinase activity of natural producers of those inducible cephalosporinases. Because the DDST was performed in parallel to the AST, results were obtained 48 h later. The DDST was considered to be the reference standard for the detection of ESBL-E.

We also used molecular biology techniques to identify the ESBL genes. We used PCR to amplify DNA, which we then sequenced using bla<sub>TEM</sub>, bla<sub>SHV</sub>, and bla<sub>CTX-M</sub> primers (9).

During the study, 245 blood specimens that were collected from patients hospitalized in any unit of the hospital...
were cultured and grew GNB (Table 1). The 245 cases of bacteremia were attributed to Enterobacteriaceae (211, 86.1%), nonfermentative GNB (31, 12.7%), and anaerobic GNB (3, 1.2%) (Table 2). Three blood cultures (1.2%) were positive for 2 enterobacterial species (Table 2). Escherichia coli was the predominant enterobacterial species (118/211, 55.9%); the next most prevalent were Klebsiella pneumoniae (37/211, 17.5%) and Enterobacter cloacae (20/211, 9.5%). Pseudomonas aeruginosa (24/31, 77.4%) was the predominant non-fermentative GNB (Table 2). Anaerobic GNB belonged to the Bacteroides fragilis group (Bacillus fragilis and Bacillus vulgatus).

We identified bacteria directly from blood culture using the MALDI-TOF technique for 237 (96.7%) isolates; results corresponded to bacterial identification after culture (Table 2). Salmonella spp. (n = 5) were correctly identified at the genus level (Table 1). For the 3 positive blood cultures that contained 2 enterobacterial species (Table 2), results were noninterpretable.

ESBL-E producers (n = 47) represented 22.3% of Enterobacteriaceae. Among the 47 ESBL-E, 30 E. coli, 13 K. pneumoniae, 3 E. cloacae, and 1 Citrobacter freundii were identified from patients who were infected in the community or the hospital (detailed data not shown) (Table 1; Table 3, http://wwwnc.cdc.gov/EID/article/21/3/14-1277-T3.htm). Most of the ESBLs were of the CTX-M-type (49/51, 96.1%); CTX-M-15 was predominant (35/51, 68.6%). The proportion of ESBL producers were 35.1%, 25.4%, and 15% among K. pneumoniae, E. coli, and E. cloacae, respectively (Table 3). The ESBL NDP test perfectly identified the 47 ESBL-E pathogens (Tables 1, 3). Accordingly, a 100% correlation between intermediate susceptibility of resistance to cefotaxime and positivity of the ESBL NDP test was observed, whereas this correlation was of 76.6% and 74.4%, respectively, when ceftazidime and cefepime susceptibility results were used for this same comparison (Table 3). The ESBL NDP test gave negative results for 164 specimens that were negative for ESBL-E (Table 2). The ESBL NDP test revealed a cefotaxime-hydrolyzing enzyme that was not inhibited by tazobactam for 1 K. pneumoniae isolate that produced an acquired cephalosporinase, 3 of the 5 E. cloacae that overproduced chromosome-encoded AmpC, and 2 Bacteroides spp. strains (data not shown).

The ESBL NDP test used with blood cultures had a sensitivity of 100% (95% CI: 92.4%–100%), a specificity of 100% (95% CI: 97.7%–100%), a positive predictive value of 100% (95% CI: 99.2%–100%) and a negative predictive value of 100% (95% CI: 97.8%–100%) for the detection of ESBL-E.

**Conclusions**

Detection of ESBLs that are the main source of cephalosporin resistance in Enterobacteriaceae still relies on antibiotic susceptibility testing, results of which usually take 24–48 h. We show that the ESBL NDP test directly performed on positive blood cultures is a reliable technique to identify ESBL-E within 30 min. Although these results are promising, they should be further confirmed in other countries where the prevalence and the epidemiology of ESBL-E might be different. A strong correlation between intermediate susceptibility or resistance to cefotaxime and positivity of the ESBL NDP test was observed (Table 3). Similar correlation between resistance to cefotaxime and ESBL production in Enterobacteriaceae was obtained in the United States (12). A concomitant use of the Carba NP test (13) directly from blood culture will also identify carbapenemase producers (such as K. pneumoniae carbapenemase producers) that also confer clavulanic-acid–inhibited resistance to cephalosporins.

**Table 1.** Origin of gram-negative bacilli identified in blood samples drawn from hospitalized patients and cultured by using rapid detection methods for bacterial blood cultures*

<table>
<thead>
<tr>
<th>Hospital department</th>
<th>No. gram-negative bacilli</th>
<th>No. (%) Enterobacteriaceae</th>
<th>No. (%) nonfermenting bacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>E. coli</td>
<td>Other</td>
</tr>
<tr>
<td>Cardiology</td>
<td>5</td>
<td>4 (80)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Digestive surgery</td>
<td>9</td>
<td>8 (89)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>Orthopedic surgery</td>
<td>3</td>
<td>3 (100)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>2</td>
<td>2 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Gerontology</td>
<td>9</td>
<td>7 (78)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Gynecology-obstetric</td>
<td>3</td>
<td>2 (67)</td>
<td>0</td>
</tr>
<tr>
<td>Hepato-gastroenterology</td>
<td>20</td>
<td>18 (90)</td>
<td>8 (44)</td>
</tr>
<tr>
<td>Emergency</td>
<td>40</td>
<td>37 (93)</td>
<td>30 (81)</td>
</tr>
<tr>
<td>Infectious diseases</td>
<td>28</td>
<td>25 (89)</td>
<td>16 (64)</td>
</tr>
<tr>
<td>Nephrology</td>
<td>28</td>
<td>21 (75)</td>
<td>8 (38)</td>
</tr>
<tr>
<td>Neurology</td>
<td>8</td>
<td>8 (100)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>Pediatric unit</td>
<td>2</td>
<td>2 (100)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Pneumology</td>
<td>4</td>
<td>3 (75)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Intensive care unit</td>
<td>67</td>
<td>57 (85)</td>
<td>34 (52)</td>
</tr>
<tr>
<td>Rhumatology</td>
<td>3</td>
<td>2 (67)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Urology</td>
<td>14</td>
<td>12 (86)</td>
<td>8 (67)</td>
</tr>
<tr>
<td>Total</td>
<td>245</td>
<td>211 (86)</td>
<td>118 (56)</td>
</tr>
</tbody>
</table>

*ESBL, extended spectrum β-lactamase.
DISPATCHES

This inexpensive ESBL NDP test might be implemented worldwide. It may optimize rapid choices of antibiotics for treating bloodstream infections. It may also contribute to avoidance of overuse of carbapenems. Finally, a rapid detection of ESBL-E coupled with bacterial species identification will enhance identification of ESBL in species likely to be the source of nosocomial outbreaks (K. pneumoniae, Enterobacter spp.) and facilitate implementation of a rapid strategy for containment (14).

This work was funded by grants from the INSERM (UMR914), France, and the University of Fribourg, Switzerland.

An international patent form for the ESBL NDP test has been filed on behalf of INSERM Transfert (Paris, France).

Dr. Dortet is an associate professor of medical microbiology, South-Paris University, Paris. His main interests include the genomics and molecular epidemiology of resistance in gram-negative rod-shaped bacteria.

References


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Methods Overview

MALDI-TOF MS bacterial identification directly on positive blood culture samples and definitive identification of bacterial species

One milliliter of blood culture was transferred into an 1.5-mL Eppendorf tube. Fifty microliters of Triton 10% was added in the tube that was then briefly vortexed. After a centrifugation step at 13,000 × g for 2 min, the supernatant was discarded and the bacterial pellet was resuspended in 500 µL of distilled water. After a second centrifugation step at 13,000 × g for 2 min, the supernatant was discarded and the bacterial pellet was resuspended into 50 µL of 100% ethanol (Thermo Scientific, Villebon-sur-Yvette, France). One drop of this suspension was spotted in duplicate on the disposable plate. The dried spots were overlaid with 1 µL of the matrix solution (VITEK-MS CHCA; bioMérieux, La Balme-les-Grottes, France) and air-dried for 1–2 min at room temperature, as recommended by the manufacturer. The loaded slide was then inserted into the VITEK MS system. Spectra were analyzed and identifications were calculated automatically by the advanced spectrum classifier algorithm provided by the manufacturer. Definitive identification retained as the gold standard was obtained with cultured bacteria using the MALDI-TOF MS bacterial identification (VITEK-MS; bioMérieux) and the Api20E biochemical gallery (bioMérieux).
Detailed Methods

Rapid Detection of ESBL Activity in *Enterobacteriaceae*

**ESBL NDP Test Directly from Blood Cultures**

**Protocol**

1. Transfer 0.5 mL of *Enterobacteriaceae*-positive blood culture (using a syringe with needle) to each of 3 Eppendorf tubes, size 1.5 mL (tubes A, B, and C).

2. Add 50 µL of a solution of Triton 10% (vol/vol).

3. Vortex.

4. Incubate 5 min at room temperature.

5. Centrifuge at 13,000 × g for 2 min.

6. Discard the supernatant.

7. Resuspend the bacterial pellet in 500 µL of distilled water.

8. Check that bacterial colonies have been properly resuspended. If needed, mix up and down with a pipette.

9. Centrifuge at 13,000 × g for 2 min.

10. Discard the supernatant.

11. Resuspend the bacterial pellet in 100 µL of 20 mmol/L Tris-HCl lysis buffer (B-PERII, Bacterial Protein Extraction Reagent; Thermo Scientific, Pierce).

12. Check that bacterial colonies have been correctly resuspended. If necessary, mix up and down with a pipette.

13. Add 10 µL of a tazobactam concentrated solution (40 mg/mL) in the tube C.

14. Add (i) 100 µL of the revelation solution (Solution R) in the tube A and (ii) 100 µl Solution R + cefotaxime 6 mg/mL in the tubes B and C.

15. Incubate at 37°C for a maximum of 15 min.
16. Optical reading of the color of each tube.

**Principle of the Test:**

![Diagram](image)

**Interpretation:**

<table>
<thead>
<tr>
<th></th>
<th>No antibiotic (tube A)</th>
<th>Cefotaxime (tube B)</th>
<th>Cefotaxime + tazobactam (tube C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ESBL</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>ESBL</td>
<td>Red</td>
<td>Orange/yellow</td>
<td>Red</td>
</tr>
<tr>
<td>Cephalosporinase or Cephalosporinase + ESBL</td>
<td>Red</td>
<td>Orange/yellow</td>
<td>Orange/yellow</td>
</tr>
<tr>
<td>Non interpretable</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

ESBL-producing *E. coli*. The *Enterobacteriaceae*-positive blood culture specimens contain A) no antibiotic; B) cefotaxime; C) cefotaxime and tazobactam.
Material

- 1.5-mL Eppendorf tubes
- Cefotaxime sodium salt (Sigma-Aldrich) or cefotaxime (drug used for patient treatment).
- Tazobactam sodium salt (Sigma-Aldrich, Cat: T-2820)
- B-PERII, Bacterial Protein Extraction Reagent (Thermo Scientific, Pierce), Cat: 78260.
- Concentrated solution of Triton X-100 (Sigma-Aldrich, Cat: T-8787)
- Negative (wild-type *E. coli*) and positive (*Klebsiella pneumoniae* CTX-M-15) controls.

Preparation and Storage of Solution R

1. Prepare a concentrated solution of red phenol 0.5% w/v
2. Mix 2 mL of the concentrated red phenol solution (strongly vortex before pipetting to resuspend the solution) in 16.6 mL of distilled water
3. Adjust the pH of the solution at a value 7.8 by adding drops of a NaOH solution (1 N)

Solution R is stable at room temperature for 1 week and may be kept at −20°C for several months.

Solution R + cefotaxime (6 mg/mL) has to be prepared extemporaneously.

However, batches of cefotaxime powders may be weighted in advance and kept at 4°C for 2 weeks if solution A is not added.

Preparation and Storage of Concentrated Tazobactam Solution (40 mg/mL)

1. Add 250 µL of distilled water in 10 mg of tazobactam sodium salt (Sigma-Aldrich, Cat: T-2820)
2. Prepare aliquots of 10 µL of this concentrated solution of tazobactam (40 mg/mL)

3. Those aliquots can be stored at −20°C for 1 month