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Comparison of Screening Methods for Detection of Extended-Spectrum β-Lactamases and Their Prevalence among Blood Isolates of *Escherichia coli* and *Klebsiella* spp. in a Belgian Teaching Hospital

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Using a set of 33 well-defined extended-spectrum β-lactamase (ESBL)-producing strains of *Escherichia coli* and *Klebsiella pneumoniae*, we compared three screening methods for ESBL detection: (i) a double-disk synergy test, (ii) a three-dimensional test (both the double-disk synergy test and the three-dimensional test were performed with ceftriaxone, ceftazidime, aztreonam, and cefepime), and (iii) the Etest ESBL screen (AB Biodisk, Solna, Sweden), based on the recognition of a reduction in the ceftazidime MIC in the presence of clavulanic acid. In the double-disk test, all four indicator antibiotics scored equally and 31 of the 33 reference strains were recognized. In the three-dimensional test, ceftriaxone was the only satisfactory indicator and 30 ESBL-positive strains were detected by this antibiotic. Both systems produced two false-positive results with cefepime. With the Etest ESBL screen, 15 of 16 TEM-related and 11 of 16 SHV-related ESBL-producing strains scored positive. In 10 cases the clavulanic acid on one end of the strip interfered with the MIC determination for ceftazidime, which was read on the opposite end. This MIC had to be determined with an extra ceftazidime-only strip. No false-positive results were noted. Eighty-six blood isolates of *E. coli* and *Klebsiella* species were screened for ESBL expression by the double-disk and three-dimensional tests, both with ceftriaxone. Six strains with suspicious antibiogram phenotypes also gave positive results by the double-disk test. One *E. coli* strain remained undetected by the three-dimensional test. Identification of the enzymes suspected of being ESBLs by isoelectric focusing (all strains) and DNA sequencing (1 strain) confirmed the screening test results except for one *Klebsiella oxytoca* strain, which proved to be a hyperproducer of its chromosomal enzyme and which also had a negative Etest score. The five true ESBL producers were all confirmed by the Etest ESBL screen. Pulsed-field gel electrophoresis proved that the *E. coli* strains were unrelated, but that two of the three *K. pneumoniae* strains were closely related.

The plasmid-mediated extended-spectrum β-lactamase (ESBL) enzymes confer resistance to oxyimino-cephalosporins, such as cefotaxime, ceftazidime, and ceftriaxone, and to monobactams, such as aztreonam. These enzymes occur predominantly in *Klebsiella* species (23) and *Escherichia coli*, but they may also be present in other genera of the family *Enterobacteriaceae*, such as *Citrobacter*, *Serratia*, *Proteus*, *Salmonella*, and *Enterobacter* (4, 15, 17–19). First recognized in Germany (13, 14), they have become increasingly prevalent in Europe and are now being reported around the world (20). The ESBLs have been reviewed extensively (10, 20, 26).

Members of the family *Enterobacteriaceae* producing ESBLs constitute a serious threat to current β-lactam therapy (10). Moreover, in vitro detection of ESBL expression has proved to be difficult because many of these strains are reportedly susceptible to the widely used and tested broad-spectrum β-lactams cefotaxime and ceftriaxone (12, 27). Therefore, specific detection methods, such as the double-disk test (11) and the three-dimensional test (27), have been developed. Double-disk testing functions via the placement of a ceftazidime disk close (20 or 30 mm) to an amoxicillin-clavulanate disk on a plate inoculated with the test organism. Enhancement of the zone of inhibition around the cephalosporin disk toward the clavulanate-containing disk indicates the presence of ESBLs (11). The sensitivity, strongly depending on the precise placement of the disks, has been reported by Thomson and Sanders (27) to be less than optimal. In the same study, the investigators reported that the three-dimensional test has a higher sensitivity.

A commercially available product for the detection of ESBL expression has been introduced by AB Biodisk (Solna, Sweden): the ESBL screening Etest strip. This Etest strip has a stable concentration gradient for ceftazidime on one end and a gradient of ceftazidime plus a fixed concentration of clavulanate on the other end. The method is based on the recognition of a reduction in ceftazidime MICs in the presence of 4 mg of clavulanic acid per liter.

In the study described here we compared the reliability of the double-disk synergy test, the three-dimensional test in a modified format, and the Etest ESBL screening test for the detection of ESBL expression with a set of well-documented ESBL-producing strains. We also determined the prevalence in our hospital of strains producing ESBLs among *E. coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* isolates from blood cultures.

MATERIALS AND METHODS

Bacterial strains. A collection of 38 reference strains of *E. coli* and *K. pneumoniae* were kindly provided by A. Medeiros (Providence, R.I.) and W. Goessens (Rotterdam, The Netherlands). The following β-lactamases, identified by

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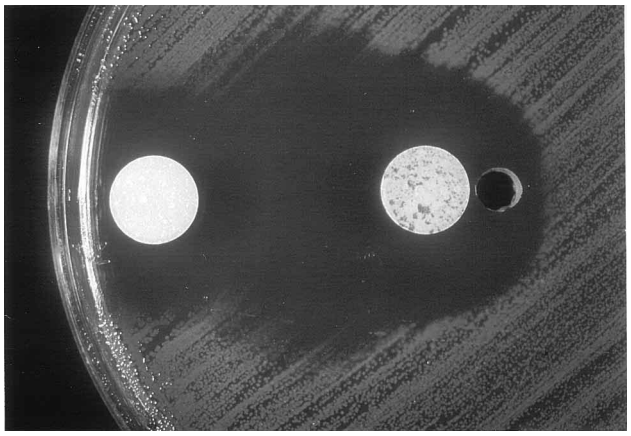

FIG. 1. Positive double-disk synergy test. The central disk contains aztreonam, and the disk on the border of the plate contains amoxicillin plus clavulanic acid. An extension of the inhibition zone around the aztreonam disk toward the clavulanate-containing disk can be observed. The well close to the aztreonam disk contains the inoculum for the three-dimensional test, which, in this case, is negative (compare Fig. 1 to Fig. 2).

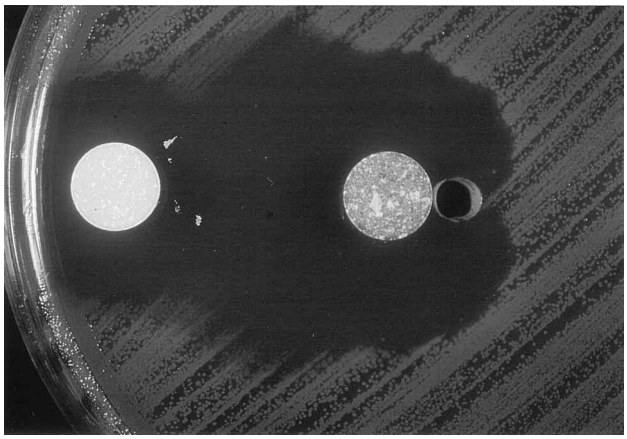

FIG. 2. Positive three-dimensional test. The central disk contains cefepime. The cup alongside the central disk is filled with the three-dimensional test inoculum (see text). Growth of the test organism appears behind the cup and reaches this cup, so that a heart-shaped distortion of the inhibition zone around the cefepime disk can be observed.

isoelectric focusing and DNA sequencing, were expressed: TEM-1 (one strain), TEM-2 (two strains), TEM-3 (eight strains), TEM-4 (two strains), TEM-5 (one strain), TEM-6 (one strain), TEM-7 (one strain), TEM-8 (one strain), TEM-9 (one strain), TEM-12 (one strain), SHV-1 (two strains), SHV-2 (three strains), SHV-3 (six strains), SHV-4 (six strains), SHV-5 (one strain), and MIR-1 (one strain). An additional set of eight wild-type strains of *E. coli* and *K. pneumoniae* that were isolated in our laboratory and that had susceptibility patterns not suggesting the production of ESBLs were also included.

The prevalence study concerned 86 strains isolated from blood cultures during 1994, including *E. coli* ($n = 64$; 74.4%), *K. pneumoniae* ($n = 13$; 15.1%), and *K. oxytoca* ($n = 9$; 10.5%). Only one strain of a given species and susceptibility pattern per patient was included.

Antimicrobial agents. Disks containing ceftriaxone (30 μg), ceftazidime (30 μg), aztreonam (30 μg), cefepime (30 μg), and amoxicillin-clavulanic acid (30 and 15 μg, respectively) were obtained from Rosco (Neo-Sensitabs, Rosco, Taastrup, Denmark). The Etest ESBL strips were obtained from AB Biodisk.

Susceptibility tests. Disk diffusion susceptibility testing was performed on Mueller-Hinton II agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Test strains were preincubated in brain heart infusion broth (Becton Dickinson Microbiology Systems) at 37°C to an optical density equal to that of a 0.5 McFarland turbidity standard. This suspension was then used to inoculate Mueller-Hinton II agar plates by swabbing them with a cotton swab. The results were interpreted by using the following instructions of the disk manufacturer: For ceftriaxone, ceftazidime, and cefepime, breakpoints are ≥20 mm (susceptible) and ≤16 mm (resistant); for aztreonam, breakpoints are ≥23 mm (susceptible) and ≤19 mm (resistant).

Both the double-disk test and the three-dimensional test were performed on the same Mueller-Hinton II agar plate for each antibiotic which was used for the detection of ESBL production. Each plate was inoculated with the test strain in the same way as for the disk diffusion procedure, and a disk of ceftazidime, ceftriaxone, cefepime, or aztreonam was placed in the center of the agar plate. For the three-dimensional test, a cylindrical plug of agar (diameter, 4 mm) 2 mm from the antibiotic disk was removed. This cup was then filled with 30 μl of the three-dimensional test inoculum, consisting of a milky suspension (McFarland no. 5 turbidity standard) of the test strain in 0.5 ml of brain heart infusion broth, which was preincubated at 37°C alongside the tube for the disk diffusion inoculum. For the double-disk synergy test, a disk of amoxicillin-clavulanic acid was placed on the agar surface opposite the cup, 30 mm (center to center, as recommended by Jarlier et al. [11]) from the central antibiotic disk.

A clear-cut extension of the inhibition zone around the β-lactam antibiotic disk toward the clavulanic acid-containing disk was interpreted as a positive double-disk synergy test result with this particular β-lactam (Fig. 1). A three-dimensional test result was positive when the inhibition zone around the β-lactam antibiotic disk was distorted in such a way that growth of the test organism appeared within the zone, behind the cup, and fully reaching this cup (Fig. 2). This is due to enzymatic inactivation of the test antibiotic by the β-lactamase diffusing through the agar in the vicinity of the three-dimensional test inoculum, resulting in a gradually decreasing diameter of the inhibition zone toward the cup. Occasionally, a subtle distortion of the inhibition zone toward the well filled with the three-dimensional test inoculum was observed. This is caused by disruption of the indicator antibiotic diffusion through the agar at the well and is interpreted as a negative test result. When inhibition zones around a β-lactam antibiotic disk were small or absent, the three-dimensional test was repeated by an indirect procedure, in which the agar surface was inoculated with the fully susceptible *E. coli* ATCC 25922 strain and the cup in the agar was inoculated with a high inoculum of the test organism. The result of this test was interpreted in the same way as that of the direct method.

The Etest ESBL strip carries two gradients: on the one end, ceftazidime (0.5 to 32 μg/ml), and on the opposite end, ceftazidime (0.125 to 8 μg/ml) plus clavulanic acid (4 μg/ml). The test was performed by following the manufacturer's instructions. Briefly, after overnight growth on brain heart infusion agar, the organisms were suspended in saline to a turbidity equal to that of a 0.5 McFarland turbidity standard. This suspension was then used to inoculate Mueller-Hinton II agar plates by swabbing them with a cotton swab. After drying, the Etest strips were placed on the plates and the plates were incubated overnight in air at 37°C. The MICs on both ends of the strip were interpreted as the point of intersection of the inhibition ellipse with the Etest strip edge (Fig. 3 and 4). According to the manufacturer, a ratio of ceftazidime MIC/ceftazidime-clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL enzymes.

Identification of β-lactamases. β-Lactamases were provisionally identified by isoelectric focusing by using alignment with well-characterized β-lactamases, the ability to hydrolyze cefotaxime in an agar overlay, and susceptibility to inhibition by clavulanate and cloxacillin (1, 22). Further enzyme identification was carried out by DNA sequencing. The DNA was prepared by heating the organisms to 95°C in distilled water for 10 min (6).

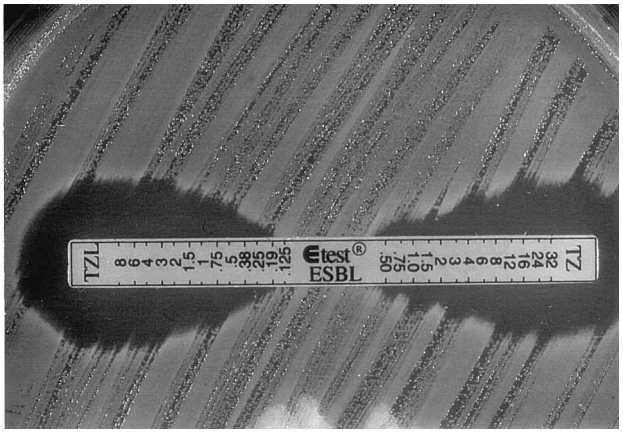

FIG. 3. Negative Etest ESBL screen test. The MIC of ceftazidime (<0.50 μg/ml) can be read at the end of the strip marked "TZ." On the opposite end, marked "TZL," the MIC of ceftazidime plus clavulanic acid (0.19 μg/ml) can be determined. This results in a ratio of the ceftazidime MIC/ceftazidime-clavulanate MIC equal to or less than 2. The cutoff value of 8 is not reached, and so the test organism is not suspected of being an ESBL producer.

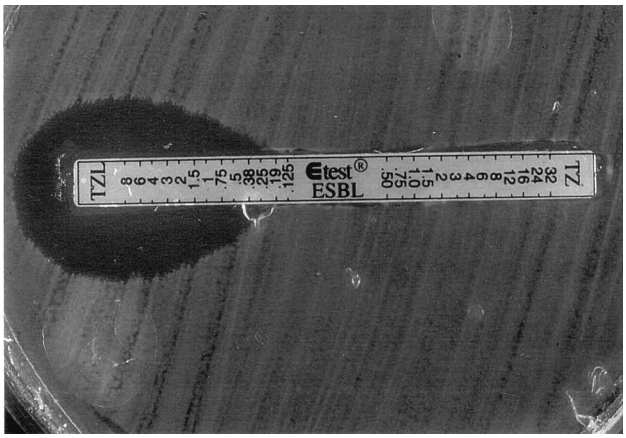

FIG. 4. Positive Etest ESBL screen test. The MIC of ceftazidime (>32 μg/ml) can be read on the end of the strip marked "TZ." On the opposite end, marked "TZL," the MIC of ceftazidime plus clavulanic acid (0.19 μg/ml) can be determined. This results in a ratio of the ceftazidime MIC/ceftazidime-clavulanate MIC equal to or greater than 253. The cutoff value of 8 is reached, and so the test organism is suspected of being an ESBL producer.

PCR (8, 16) was used to amplify a TEM-specific product by using the following primers: forward primer, 5′-TCGGGGAAATGTGCG-3′, bases 91 to 105, and reverse primer, 5′-TGCTTAATCAGTGAGGCACC-3′, bases 1062 to 1042 (numbering according to Sutcliffe [24]). PCR conditions were those suggested by Perkin-Elmer, in which AmpliTaq DNA polymerase and 2.0 mM $MgCl_2$ were used. All control DNA was prepared in the same manner as the DNA templates used for the clinical isolate. The controls for PCR were as follows: positive control, TEM-1, and negative control, *E. coli*, C 600. A second negative control was water, which was added at the time and place of template addition, thereby controlling for crossover contamination. Amplification was performed with the Perkin-Elmer DNA thermocycler 480 with the following cycling parameters for 25 cycles: denaturation at 96°C for 30 s, annealing at 50°C for 15 s, and extension at 72°C for 2 min. A single band on a 2% agarose gel was observed in the PCRs with either the positive control with TEM-1 or the clinical isolate with TEM-6. All other PCRs were devoid of any band, specific or nonspecific. The PCR product was sequenced by automated PCR cycle sequencing by dye-terminator chemistry with a DNA stretch sequencer from Applied Biosystems. The sequence of the β-lactamase gene was compared to the nucleotide sequences of Sutcliffe (24) (TEM-1) and Goussard et al. (5) (TEM-6).

Analysis of chromosomal DNA by PFGE. All reagents were from Sigma Chemical Co. unless otherwise noted. Strains were grown overnight at 37°C in 5% CO_2 on Columbia agar (GIBCO, Life Technologies, Paisley, Scotland) supplemented with 5% defibrinated horse blood. One loop of cells was washed three times in 1 ml of EET buffer [100 mM disodium EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM Tris-HCl (pH 8.0)]. The pelleted cells were resuspended in EC buffer (6 mM Tris-HCl [pH 7.6], 1 M NaCl, 100 mM disodium EDTA [pH 8.0], 0.5% polyoxyethylene 20 cetyl ether [Brij 58], 0.2% sodium deoxycholate, 0.5% *N*-lauroylsarcosine) and were adjusted to a density of 4×10^9 CFU/ml, mixed with an equal volume of 1.6% (wt/vol of EC buffer) low-melting-temperature preparative-grade agarose (Bio-Rad Laboratories), and pipetted into the plug mold. The solidified plugs were transferred to 0.5 ml of EC lysis buffer (2.88 mg of lysozyme per ml of EC buffer), and the mixture was incubated overnight at 37°C. The lysis buffer was replaced by a protein digestion buffer (3.3 mg of pronase E and 1.6% [wt/vol] sodium dodecyl sulfate/ml of EET buffer), and the mixture was incubated overnight at 37°C. Before restriction digestion the agarose plugs were washed four times for 1 h each time in EET buffer, twice for 1 h each time in $T_{10}E_{0.1}$ buffer (10 mM Tris-HCl, 0.1 mM disodium EDTA [pH 8.0]), and 1 h in the appropriate restriction buffer at room temperature.

Macrorestriction of the genomic DNA was carried out overnight at 37°C in 250 μl of fresh restriction buffer containing 30 U of *Spe*I (Eurogentec) for *Klebsiella* species (21) and 30 U of *Xba*I (Eurogentec) for *E. coli* (28). The digestion reaction was stopped by the addition of 0.5 ml of 0.5 M disodium EDTA (pH 8.0). The chromosomal restriction fragments were analyzed by pulsed-field gel electrophoresis (PFGE) in a CHEF MAPPER system (Bio-Rad Laboratories) by loading small pieces of the plugs (5 by 2 by 0.5 mm) into the slots of an agarose, pulsed-field-certified, Bio-R-L 1% gel (Bio-Rad Laboratories) prepared in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM disodium EDTA [pH 8.0]) and sealed with the same agarose. Electrophoresis was performed in 2 liters of 0.5× TBE equilibrated at 14°C at a constant voltage of 6 V/cm, with pulse times ramped linearly from 5 to 10 s for 10 h, from 10 to 30 s for 4 h and from 30 to 40 s for 4 h.

The *Sma*I (Imtec)-digested genome of *Staphylococcus aureus* NCTC 8325 was used as a molecular size standard (25). The gels were stained with ethidium bromide solution (5 mg/liter) for 30 min, destained in distilled water for 60 min, rinsed, and photographed.

RESULTS

Disk diffusion susceptibility tests. No pattern of reduced susceptibility (resistance or intermediate susceptibility) to expanded-spectrum cephalosporins and/or aztreonam that was predictive of ESBL production could be identified (Table 1). Even when test results with ceftriaxone, ceftazidime, and aztreonam were grouped, only 52% of the ESBL-producing strains were detected. ESBL production was not detected in some strains producing TEM-3, TEM-4, TEM-12, SHV-2, SHV-3, SHV-4, or SHV-5. Although ceftazidime was the best single indicator antibiotic for ESBL production, all strains producing TEM-4, TEM-12, SHV-2, SHV-3, or SHV-5 remained undetected by it.

Three-dimensional tests. For the three-dimensional test, we did not apply the technique described by Thomson and Sanders (27) involving the application of the three-dimensional test inoculum into a circular slit in the agar, because this method requires a customized low-speed turntable. We also experimented with 30-mm-long straight slits alongside each antibiotic disk. However, filling these slits homogeneously with a bacterial suspension and without overflow onto the agar surface was difficult and fastidious.

In the modified three-dimensional test, ceftriaxone proved, by far, to be the best indicator of ESBL production (Table 2). Only TEM-6, TEM-7, and TEM-12 were not detected. The direct method, with ceftriaxone, was unsuccessful with four strains because of small inhibition zones. By the indirect pro-

TABLE 1. Disk diffusion test results for 33 ESBL-producing and 13 non-ESBL-producing *E. coli* and *Klebsiella* strains

Type of strain	% Strains intermediate or resistant to the following[a]:				
	CTR	CAZ	AZT	CTR, CAZ, or AZT	CEP
ESBL producers					
TEM related (n = 16)	25	62	38	62	19
SHV related (n = 16)	12	31	25	38	0
MIR-1 (n = 1)	100	100	100	100	0
Total (n = 33)	21	48	33	52	9
Non-ESBL producers					
TEM-1 or TEM-2 (n = 3)	0	0	0	0	0
SHV-1 (n = 2)	0	0	0	0	0
Wild type (n = 8)	0	0	0	0	0

[a] CTR, ceftriaxone; CAZ, ceftazidime; AZT, aztreonam; CEP, cefepime.

TABLE 2. Results of the three screening methods for ESBL production with strains producing TEM-related, SHV-related, and MIR-related, ESBLs and negative control and wild-type strains

Type of β-lactamase produced or type of strain	Three-dimensional test result with the following[a]:				Double-disk test result with the following:				Etest results			
	CTR	CAZ	AZT	CEP	CTR	CAZ	AZT	CEP	MIC (μg/ml) of CAZ	MIC (μg/ml) of CAZ + CL	MIC ratio	Test score[b]
TEM-3	+	0	0	0	+	+	+	+	2[c]	0.125	16	+
TEM-3	+[d]	0[d]	0[d]	0[d]	+	+	+	+	>32	0.38	≥126	+
TEM-3	+[d]	0[d]	0[d]	+	+	+	+	+	>32	0.5	≥96	+
TEM-3	+	0[d]	0[d]	+	+	+	+	+	>32	0.19	≥253	+
TEM-3	+	0	0	+	+	+	+	+	6	0.125	48	+
TEM-3	+[d]	0[d]	0[d]	0[d]	+	+	+	+	>32	0.5	≥96	+
TEM-3	+	0[d]	0	+	+	+	+	+	16	0.125	128	+
TEM-3	+	0	+	0	+	+	+	+	2[c]	<0.125	≥21	+
TEM-4	+	0	0	0	+	+	+	+	3[c]	<0.125	≥32	+
TEM-4	+	0	0	0	+	+	+	+	3[c]	<0.125	≥32	+
TEM-5	+	0[d]	0	0	+	+	+	+	>32	0.38	≥126	+
TEM-6	0	0[d]	0	0	+	+	+	+	>32	0.19	≥253	+
TEM-7	0	0[d]	0	+	+	+	+	+	>32	0.125	≥384	+
TEM-8	+	0[d]	0	+	+	+	+	+	>32	0.5	≥96	+
TEM-9	+	0[d]	0[d]	+	+	+	+	+	>32	0.75	≥64	+
TEM-12	0	0	0	0	0	0	0	0	<0.5	0.38	≤1	0
SHV-2	+	0	0	0	+	+	+	+	0.38[c]	<0.125	≥4	?
SHV-2	+	0	0	+	+	+	+	+	>32	0.125	≥384	+
SHV-2	+	0	0	+	+	+	+	+	0.5	0.125	4	0
SHV-3	+	0	0	0	+	+	+	+	0.5[c]	<0.125	≥5	?
SHV-3	+	0	0	+	+	+	+	+	0.75[c]	<0.125	≥8	+
SHV-3	+	0	0	+	+	+	+	+	1.5	0.25	6	0
SHV-3	+	+	0	+	+	+	+	+	2	0.25	8	+
SHV-3	+	0	+	+	+	+	+	+	6	0.25	24	+
SHV-3	+	+	0	+	+	+	+	+	0.5	0.125	4	0
SHV-4	+	+	+	+	+	+	+	+	128[c]	<0.125	≥1,362	+
SHV-4	+	+	0	0	+	+	+	0	1[c]	0.125	8	+
SHV-4	+	+	+	0	+	+	+	+	16[c]	0.125	128	+
SHV-4	+	0[d]	0[d]	0	+	+	+	+	>32	0.125	≥384	+
SHV-4	+	0[d]	0[d]	+	+	+	+	+	>32	0.19	≥253	+
SHV-4	+	+[d]	0	+	+	+	+	+	>32	<0.125	≥511	+
SHV-5	+	0	0	0	+	+	+	+	>32	<0.125	≥511	+
MIR-1	+[d]	0	0[d]	+	0	0	0	0	>32	>8	?	?
TEM-1	0	0	0	0	0	0	0	0	<0.5	0.125	≤3	0
TEM-2	0	0	0	+	0	0	0	+	<0.5	0.125	≤3	0
TEM-2	0	0	0	+	0	0	0	+	<0.5	0.125	≤3	0
SHV-1	0	0	0	0	0	0	0	0	<0.5	0.125	≤3	0
SHV-1	0	0	0	0	0	0	0	0	<0.5	0.125	≤3	0
Wild type-1 (Ec[e])	0	0	0	0	0	0	0	0	<0.5	0.19	≤2	0
Wild type-2 (Kp[f])	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?
Wild type-3 (Ec)	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?
Wild type-4 (Ko[g])	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?
Wild type-5 (Ec)	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?
Wild type-6 (Ko)	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?
Wild type-7 (Ko)	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?
Wild type-8 (Kp)	0	0	0	0	0	0	0	0	<0.5	<0.125	?	?

[a] CTR, ceftriaxone; CAZ, ceftazidime; CAZ + CL, ceftazidime with clavulanate; AZT, aztreonam; CEP, cefepime; 0, negative test result; +, positive test result.
[b] An MIC ratio of ≥8 corresponds to a positive test score; an MIC ratio of <8 corresponds to a negative test score.
[c] Testing with separate ceftazidime-only strip was required.
[d] Indirect testing necessary.
[e] Ec, *E. coli.*
[f] Kp, *K. pneumoniae.*
[g] Ko, *K. oxytoca.*

cedure, however, with the fully susceptible *E. coli* ATCC 25922 strain, these four strains produced a positive result. Cefepime, aztreonam, and ceftazidime detected 7, 1, and 0 of the 16 TEM-related ESBL enzymes, respectively. In 2, 5, and 10 cases, indirect testing proved necessary, but it never produced a positive result with cefepime, aztreonam, and ceftazidime, respectively. These three antibiotics scored better with SHV-related ESBL enzymes and MIR-1. Still, even with cefepime, the best indicator of these three, 6 of 17 of these types of ESBL enzymes remained undetected. With aztreonam and ceftazidime, indirect testing was necessary for three strains, of which one produced a positive result with ceftazidime. With cefepime, indirect testing was never required.

Of the TEM-1- and TEM-2- and SHV-1-producing strains,

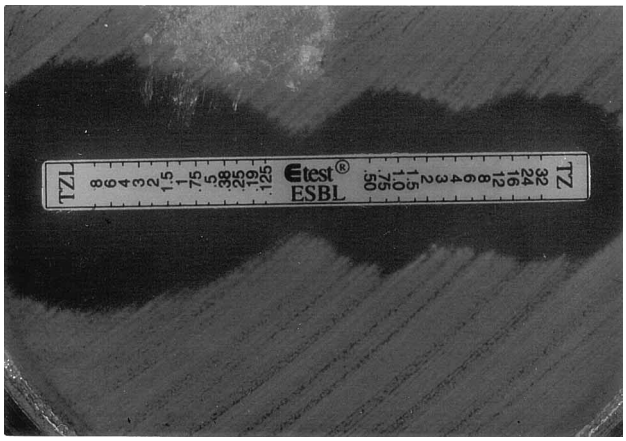

FIG. 5. Unreadable Etest ESBL screen test. The MIC of ceftazidime, which should be read on the end of the strip marked "TZ," cannot be determined because the inhibition ellipse is distorted by the action of clavulanic acid diffusing from the opposite end of the strip.

only the two TEM-2 producers gave a positive result in this test, although only with cefepime (Table 2). None of the additional wild-type strains scored positive by the three-dimensional method.

Double-disk tests. All TEM-related ESBL enzymes except TEM-12 were detected by the double-disk test with the recommended 30-mm spacing (Table 2). Reducing the spacing distance to 20 mm did not resolve this false-negative result. Identical results were obtained with the four antibiotics, and ceftriaxone was selected for screening of the blood isolates. The same applied to SHV-related ESBL enzymes, except that cefepime did not detect one SHV-4 enzyme (Table 2). Because MIR-1 is a clavulanic acid-resistant β-lactamase, it also remained undetected.

The two ESBL-negative strains producing positive three-dimensional test results with cefepime were also positive in the double-disk test with this antibiotic (Table 2). None of the 8 wild-type strains were positive by the double-disk test.

Etests. The special Etest proved to be very effective for the detection of TEM-related ESBL enzymes (Table 2). Only TEM-12, also undetected by the double-disk test and the three-dimensional method, was missed. In four cases the MIC of ceftazidime could not be read because the inhibition zone was distorted by the clavulanic acid diffusing from the opposite end of the strip (Fig. 5). In these cases the MIC of ceftazidime had to be measured with a separate conventional strip containing only ceftazidime.

However, for the SHV-related ESBL enzymes, this method proved less satisfactory: only 11 of the 16 strains producing this type of enzyme were detected (Table 2). In three cases the MIC ratio was below 8, and in two cases the MIC ratio was inconclusive. An example of the latter is an MIC ratio of ≥4, which means that it can be 4 or 6, both giving a negative test score, but also 8 or greater than 8, giving positive test scores. For the MIR-1 producer the MIC ratio could not be determined (both MICs were above the upper limit of both scales).

All TEM-1- and TEM-2- and SHV-1-producing strains and one wild-type strain gave negative test results (Table 2). For the seven other wild-type strains the MIC ratio could not be determined (both MICs were below the lower limit of both scales) and the test proved inconclusive.

Detection of ESBL-producing strains among blood isolates. On the basis of their susceptibility patterns, i.e., reduced susceptibility to at least one expanded-spectrum cephalosporin and/or to aztreonam, 6 of the 86 blood isolates were suspected of producing ESBL enzymes: *E. coli*, 2 strains; *K. pneumonia*, 3 strains; and *K. oxytoca*, 1 strain.

All isolates were screened by a combination of the double-disk and three-dimensional tests on one agar plate with ceftriaxone as the indicator antibiotic. Additionally, with all screen-positive strains both tests were repeated with the other three antibiotics, and an Etest ESBL screen was performed.

Eighty of the 86 blood isolates remained negative by the double-disk test. The six strains having a suspicious antibiogram scored positive by this method with all four antibiotics, except for ceftazidime with the *K. oxytoca* strain (Table 3).

Five of the six blood isolates suspected of producing ESBL enzymes on the basis of their susceptibility patterns and positive scores in the double-disk test were the only strains positive in the three-dimensional test (two strains required indirect testing with all indicators). One strain (*E. coli* 8D128) remained negative in the three-dimensional test with ceftriaxone, as well as with every other indicator antibiotic (Table 3).

Of the six blood isolates presumptively identified as ESBL producers by the three-dimensional test and/or double-disk test, only the *K. oxytoca* strain, negative by both tests with ceftazidime, was also negative by the Etest.

The enzymes suspected of being ESBLs detected in the six blood isolates were further identified on the basis of isoelectric focusing (all strains) and DNA sequencing (*E. coli* 8D128). The following final identification results were obtained: *E. coli* 8A60, SHV-5 (pI 8.0); *E. coli* 8D128, TEM-6 (pI 5.9); *K.*

TABLE 3. Test results of the three screening methods for ESBL production with positive blood isolates[a]

Strain no.	Species[b]	Double-disk test result with the following:				Three-dimensional test result with the following:				Etest results			
		CTR	CAZ	AZT	CEP	CTR	CAZ	AZT	CEP	MIC (μg/ml) of CAZ	MIC (μg/ml) of CAZ + CL	MIC ratio	Test score[b]
8A60	Ec	+	+	+	+	+	0	0	+	16	0.125	128	+
8D128	Ec	+	+	+	+	0	0	0	0	>32	<0.125	≥511	+
7C138	Kp	+	+	+	+	+[c]	0[c]	0[c]	0[c]	>32	0.38	≥126	+
8B128	Kp	+	+	+	+	+	0	0	+	>32	0.125	≥384	+
8C261	Kp	+	+	+	+	+	0	0	+	>32	0.125	≥384	+
7D5	Ko	+	0	+	+	+[c]	0[c]	+[c]	+[c]	0.75	0.125	6	0

[a] CTR, ceftriaxone; CAZ, ceftazidime; CAZ + CL, ceftazidime plus clavulanate; AZT, aztreonam; CEP, cefepime; 0, negative test result; +, positive test result; Ec, *E. coli*; Kp, *K. pneumoniae*; Ko, *K. oxytoca*.

[b] An MIC ratio of ≥8 corresponds to a positive test score; an MIC ratio of <8 corresponds to a negative test score.

[c] Indirect testing necessary.

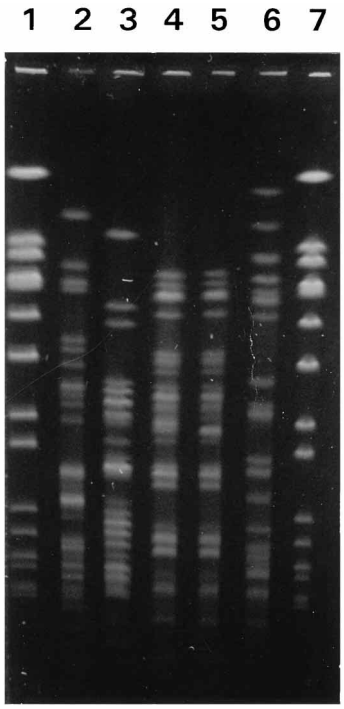

FIG. 6. PFGE patterns of ESBL-positive blood isolates. Lanes: 1, *S. aureus* NCTC 8325; 2, *E. coli* 8D128; 3, *E. coli* 8A60; 4, *K. pneumoniae* 7C138; 5, *K. pneumoniae* 8C261; 6, *K. pneumoniae* 8B128; 7, *S. aureus* NCTC 8325. Only *K. pneumoniae* 7C138 and 8C261 (lanes 4 and 5, respectively) proved to be closely related by their *Spe*I digestion patterns.

pneumoniae 7C138, SHV-5 (pI 8.0); *K. pneumoniae* 8B128, SHV-5 (pI 8.0); *K. pneumoniae* 8C261, SHV-5 (pI 8.0); and *K. oxytoca* 7D5 (pI 6.5), hyperproduced chromosomal enzyme.

On the basis of PFGE the *E. coli* strains were unrelated, while two of the three *K. pneumoniae* strains (strains 7C138 and 8C261) were closely related, as revealed by their almost indistinguishable PFGE *Spe*I digestion patterns (Fig. 6).

DISCUSSION

Standard disk diffusion susceptibility tests detected only 48% of the ESBL-producing reference strains by their reduced susceptibility to ceftazidime. This percentage could not be significantly increased by combining the results with those for other indicator antibiotics. Among the five ESBL-producing blood isolates, only three strains were clearly resistant to ceftazidime (two of these were also resistant to aztreonam). Thus, in agreement with other studies (9, 12, 27), it is clear that for the detection of ESBL enzymes, additional tests are required.

Thirty-one of 33 ESBL-producing strains were detected by the double-disk test with ceftriaxone, leaving only the TEM-12 and the MIR-1 producers undetected. The three-dimensional test with ceftriaxone was slightly less sensitive (three strains scored negative), but it was able to pick up the MIR-1-producing strains. Because of small or absent inhibition zones, testing had to be repeated by the indirect procedure for three TEM-3 producers and the MIR-1-positive strain, resulting in a positive test result for each. When we excluded the producer of MIR-1, a β-lactamase no longer considered an ESBL since it has been shown to be an AmpC derivative, the sensitivities of the double-disk and three-dimensional tests were 96.9 and 90.6%, respectively. This is not in agreement with the results of a similar study by Thomson and Sanders (27), which reported, for TEM- and SHV-related ESBLs, a higher sensitivity for the three-dimensional test (92.3%) than for the double-disk test (84.6%). However, we must stress the facts that (i) in our study Rosco tablets instead of paper disks were used because Rosco tablets are readily available in the majority of Belgian diagnostic laboratories, (ii) the loads of amoxicillin and clavulanic acid on Rosco tablets (30 and 15 μg, respectively) differ from those on standard paper disks (20 and 10 μg, respectively), and (iii) we changed the procedure for the three-dimensional test significantly. Finally, the possibility that the TEM-12-producing strain lost its β-lactamase during manipulations in the laboratory cannot be excluded.

Cefepime produced false-positive results by both the double-disk test and the three-dimensional test with both TEM-2-producing strains. A possible explanation could be a combination of the facts that (i) cefepime is more readily hydrolyzed by many β-lactamases, (ii) this β-lactamase is produced in high quantities, and (iii) it is noted that cefepime penetration into bacterial cells is slower in vitro than in vivo.

Despite previous positive reports on the sensitivity of the Etest ESBL screening method (2, 7), this method detected only 26 of the 33 ESBL-positive strains, while for 10 strains the determination of the MIC of ceftazidime required additional tests with a ceftazidime-only strip. Considering only TEM- and SHV-like ESBLs, the Etest system detected 81.2% of the ESBL producers when an MIC ratio equal to or greater than 8, as recommended by the manufacturer, was used as an indicator for ESBL production. Cormican et al. (3) proposed that the breakpoint for ESBL positivity be lowered from 8 to 5, which is equivalent to lowering the required minimum number of $\log_2$ dilution steps by which the ceftazidime MIC must be reduced in the presence of clavulanic acid to obtain a positive score from 3 to 2.5. Use of this criterion allowed for the detection of two additional strains (one for which the MIC ratio was ≥5 and one for which the MIC ratio was 6) and increased the sensitivity to 87.5%. These results, combined with the fact that Etest strips are very expensive, made us decide to use the combination of double-disk and three-dimensional testing with ceftriaxone for further study of the prevalence of ESBL producers among blood isolates in our hospital. Nevertheless, we agree that interpretation of double-disk test results is more subjective than reading Etest results and requires very experienced investigators to obtain reliable results.

Among 86 blood isolates studied, 5 (5.8%) ESBL-producing strains were detected: 2 of 64 (3.1%) *E. coli* strains and 3 of 13 (23.1%) *K. pneumoniae* strains. In contrast to other reports (2), an alarming percentage of the *K. pneumoniae* blood isolates were ESBL producers. Although one *K. oxytoca* strain (strain 7D5) was positive by the double-disk test with ceftriaxone, further testing with ceftazidime showed negative results. Subsequent characterization confirmed that this strain is a hyperproducer of its chromosomal enzyme. Indeed, as opposed to most klebsiellae with extended-spectrum TEM and SHV enzymes, these strains remain susceptible to ceftazidime. Isoelectric focusing identified four of the five ESBLs from screen-positive isolates as SHV-5. For *E. coli* 8D128, DNA sequencing was necessary to resolve the identity of the ESBL, a TEM-6 enzyme, harbored by this strain. Three ESBL-producing strains were isolated from patients in different wards, and two ESBL-producing strains were isolated from two patients in the hematology ward. *E. coli* 8D128 (TEM-6) and *K. pneumoniae* 7C138 (SHV-5) were isolated from the blood of these patients with a 5-month time lapse. Study of the PFGE digestion patterns of the ESBL-positive blood isolates revealed a close relationship only between two *K. pneumoniae* strains, isolated on different wards, also with a 5-month time lapse. To get a

clear picture of the situation concerning ESBL-producing members of the family *Enterobacteriaceae* in our hospital, we are performing a prospective study.

We conclude that although close monitoring of the susceptibility patterns of members of the family *Enterobacteriaceae*, e.g., by using the algorithm proposed by Thomson and Sanders (26), can be useful for selecting suspect ESBL producers, a special ESBL screening test is indispensable. We propose use of the combination of double-disk and three-dimensional tests with ceftriaxone with Neo-Sensitabs as a sensitive and specific screening method for ESBLs. Use of a combination of tests makes it easier for the investigator to interpret test results.

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