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Modification of the Double-Disk Test for Detection of *Enterobacteriaceae* Producing Extended-Spectrum and AmpC β-Lactamases

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Detection of extended-spectrum β-lactamases (ESBLs) in AmpC-producing *Enterobacteriaceae* is problematic. A modification of the double-disk test (MDDT) has been developed for successful detection of ESBLs in gram-negative bacilli producing well-characterized β-lactamases as well as 212 clinical isolates of *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marcescens*, and *Citrobacter freundii*. MDDT accurately differentiated between ESBL producers and derepressed chromosomal AmpC mutants. MDDT provides a cost-effective alternative approach for clinical microbiology laboratories for routine susceptibility testing with simultaneous detection of ESBLs in *Enterobacteriaceae*.

Enterobacteriaceae producing both AmpC β-lactamases and extended-spectrum β-lactamases (ESBLs) have been increasingly reported worldwide (2–6, 12, 16, 18). Since AmpC-producing organisms can act as hidden reservoirs for ESBLs, it is important for clinical microbiology laboratories to be able to detect ESBL production in these organisms on a routine basis (9). The National Committee for Clinical Laboratory Standards (NCCLS) has published guidelines for the detection of ESBLs in clinical isolates of *Escherichia coli* and *Klebsiella* spp., but there are currently none available for other genera (10). Since high-level expression of AmpC β-lactamases may mask recognition of ESBLs (17), a unique modification of the double-disk test (MDDT) using a combination of cefepime (FEP) and piperacillin-tazobactam (TZP) was evaluated to detect ESBLs. This evaluation was carried out with well-characterized strains producing either AmpC β-lactamases and ESBLs or either β-lactamase alone. In addition, the MDDT determined the presence of an ESBL in an *E. coli* isolate that showed a negative result with the NCCLS disk confirmation test.

The following strains producing known β-lactamases were used for this study (Table 1). A total of 212 clinical isolates were also evaluated by MDDT: 94 were *E. cloacae*, 32 were *E. aerogenes*, 25 were *S. marcescens*, and 61 were *C. freundii*. The isolates were nonrepetitive (one per patient) and were obtained from clinical specimens from Universitas and Pelonomi Hospitals, Bloemfontein, South Africa, over a 9-month period during 1998 and 1999.

The susceptibility of the isolates was determined by the standard disk diffusion method as described in the NCCLS guidelines (10). Disks for the agar diffusion procedure were obtained from Becton Dickinson Microbiology Systems (Johannesburg, South Africa).

MDDT was performed on both control and clinical strains. Modifications of the original double-disk test were as follows (8). Ceftriaxone (30-μg disk) was replaced with FEP (30-μg disk), the cefotaxime (CTX) (30-μg) disk was placed 20 mm from the amoxicillin (20 μg)-clavulanate (10 μg) (AMC) disk, the aztreonam (ATM) (30 μg) disk was placed at 25 mm, the ceftazidime (CAZ) (30 μg) disk was placed at 30 mm, and the FEP (30 μg) disk was placed at 30 mm (Fig. 1). This modification was incorporated into a gram-negative template, and a piperacillin (100 μg)-tazobactam (10 μg) (TZP) disk was placed 25 mm from FEP (Fig. 1).

Sonic extracts containing β-lactamases were assessed for isoelectric points and substrate and inhibitor profiles in polyacrylamide gels as previously described (13). PCR amplification was performed to determine the presence of bla_{TEM} or bla_{SHV}, and the SHV amplicon was sequenced as previously described (13).

The MDDT detected the presence of ESBLs in all of the well-characterized strains (Table 1). The clinical strains were divided into three groups according to interpretation with MDDT and susceptibilities to CTX and CAZ (Table 2): group 1 (wild type), ESBL negative, sensitive to CTX and CAZ; group 2 (derepressed mutants), ESBL negative, resistant to CTX and CAZ; and group 3 (ESBL producers) ESBL positive, sensitive, intermediate, or resistant to CTX and CAZ.

Isoelectric focusing (IEF) was performed on the strains in groups 2 and 3. Strains representing the group 2 phenotype produced β-lactamases with pI values ranging from 8.0 to 8.9 and were inhibited by cloxacillin on IEF gels. This correlates with Bush group 1 cephalosporinases (1). The ESBL-positive group produced similar Bush group 1 cephalosporinases (Table 2) as well as additional β-lactamases with pIs of 8.2, 7.6, and 5.6 that were inhibited by clavulanate on IEF gels and showed CTX hydrolysis (0.75 μg/ml) for bands focusing at pIs

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TABLE 1. Control and clinical strains producing well-characterized β-lactamases

Strain	Organism	β-Lactamase[a]	MDDT result	Source or reference[b]
RTEM	*E. coli*	TEM-1	Negative	a
1752E	*E. coli*	TEM-2	Negative	a
J53	*E. coli*	SHV-1	Negative	a
CF204	*E. coli*	TEM-3	Positive	a
K12	*E. coli*	TEM-4	Positive	a
CF604	*E. coli*	TEM-5	Positive	a
CF804	*E. coli*	TEM-6	Positive	a
C600	*E. coli*	TEM-7	Positive	a
C600	*E. coli*	TEM-8	Positive	a
2639E	*E. coli*	TEM-9	Positive	a
C600	*E. coli*	TEM-10	Positive	a
C600	*E. coli*	TEM-16	Positive	a
CF1104	*K. pneumoniae*	TEM-24	Positive	a
PJPQ101	*E. coli*	TEM-26	Positive	a
C600	*E. coli*	SHV-2	Positive	a
J53	*E. coli*	SHV-3	Positive	a
J53-2	*E. coli*	SHV-4	Positive	a
ClaNal	*E. coli*	SHV-5	Positive	a
DH52	*E. coli*	SHV-7	Positive	a
C600	*E. coli*	MIR-1	Negative	a
NU2936	*K. pneumoniae*	MOX-1	Negative	a, b
F12	*K. pneumoniae*	FOX-1	Negative	a, b
P20	*K. pneumoniae*	LAT-1	Negative	a, b
C600	*E. coli*	BIL-1	Negative	a, b
AR15	*E. coli*	CTXM-1	Positive	a
Pit 16	*K. pneumoniae*	TEM-63	Positive	13
Pit 100	*K. pneumoniae*	SHV-2	Positive	13
Pit 82	*K. pneumoniae*	SHV-5	Positive	13
Pit 64	*E. coli*	TEM-63	Positive	13
Pit 56	*E. coli*	SHV-2	Positive	13
Pit 85	*P. mirabilis*	TEM-63	Positive	13
187	*E. aerogenes*	AmpC + SHV-3	Positive	12
184	*E. aerogenes*	AmpC + SHV-5	Positive	12
200	*E. aerogenes*	AmpC + SHV-4	Positive	12
220	*E. aerogenes*	AmpC + SHV-4	Positive	12
86	*E. aerogenes*	AmpC + TEM-3	Positive	11
94	*E. cloacae*	AmpC + SHV-2	Positive	11
142	*E. cloacae*	AmpC + TEM-3	Positive	11
029	*E. cloacae*	AmpC (WT)	Negative	11
108	*E. cloacae*	AmpC (DM)	Negative	11

[a] WT, wild type; DM, derepressed mutant.
[b] a, provided by Creighton University, Omaha, Nebr.; b, plasmid-mediated AmpC β-lactamases.

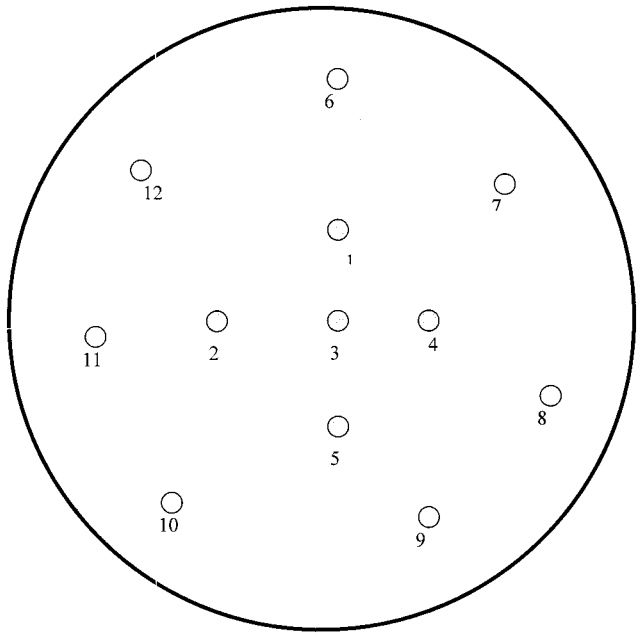

FIG. 1. MDDT and the gram-negative template. The antibiotic disk numbers represent the following antibiotics: 1, ATM; 2, FEP; 3, AMC; 4, CTX; 5, CAZ; 6, imipenem; 7, cefoxitin; 8, cefuroxime; 9, gentamicin; 10, amikacin; 11, TZP; and 12, ciprofloxacin.

of 8.2, 7.6, and 5.6. These enzymes have characteristics of Bush group 2e β-lactamases (ESBLs) (14) (Table 2).

TEM- and SHV-specific PCR was performed on DNA obtained from group 3 (Table 2). An 885-bp fragment specific for bla_{SHV} was amplified in organisms producing β-lactamases with pIs of 7.6 and 8.2, and a 971-bp fragment specific for bla_{TEM} was amplified in organisms producing β-lactamases with pI values of 5.6. Taken together, the data obtained from IEF and PCR indicate that these strains produced both AmpC β-lactamases and either SHV or TEM ESBLs. A positive test was present for the organisms in group 3 when the MDDT to ATM, FEP, and/or TZP disks was used. Therefore, the MDDT successfully differentiated between ESBL producers and overexpression of AmpC-derepressed mutants.

An additional *E. coli* isolate obtained from a urine culture, indicating a negative confirmatory ESBL disk test as recommended by the NCCLS, showed a positive MDDT. This isolate was resistant to cefoxitin and cefpodoxime, intermediate in resistance to CAZ, and sensitive to ceftriaxone. Further evaluation of this strain by IEF revealed the production of two β-lactamases consistent with the presence of Bush group 1 (pI >9.0) and group 2e (pI 7.6). The group 2e enzyme was confirmed as SHV-2 by PCR and sequencing (13, 14).

Several types of TEM and SHV ESBLs have been described in isolates of *K. pneumoniae*, *E. coli*, and *Proteus mirabilis* from South Africa (7, 13). This is the first report of TEM and SHV ESBL production in *Enterobacter* species, *C. freundii*, and *S. marcescens* isolates in South Africa. The characterization of the different enzymes was outside the scope of this study.

In this study, ESBL production among *Enterobacter* species, *C. freundii*, and *S. marcescens* was associated with high levels of resistance to trimethoprim-sulfamethoxazole and gentamicin when compared to derepressed chromosomal AmpC mutants (data not shown). Since ESBL producers express their β-lactamase genes from plasmids, these organisms may also have genes coding for resistance to additional classes of antibiotics (15). This report demonstrates the need to differentiate AmpC overproducers from ESBL-producing strains.

There are no published guidelines for the detection of ESBLs in organisms other than *E. coli* and *Klebsiella* spp. The original double-disk test, Vitek ESBL card, and E-test ESBL strips failed to detect ESBL-producing isolates of *C. freundii*, *E. cloacae*, *E. aerogenes*, *S. marcescens*, *Morganella morganii,* and *Providentia stuartii* (16, 18). The MDDT described in this study detected ESBLs in strains with well-characterized β-lactamases as well as clinical strains of *E. cloacae*, *E. aerogenes*, *C. freundii*, and *S. marcescens.* Furthermore, the MDDT was able to detect the presence of an ESBL (SHV-2) in an AmpC-producing *E. coli* isolate that failed the NCCLS ESBL disk confirmation test. This modification has been incorporated into a gram-negative template for routine susceptibility testing with the additional benefits of simultaneous detection of ESBLs. The MDDT provides a cost-effective alternative for

TABLE 2. Characteristics of β-lactamases produced by different resistant phenotypes

Resistant phenotype[a]	(no. of strains)	Susceptibility to[b]:		MDDT result	pI	CTX hydrolysis[c]	Inhibited by[d]:		Amplification with:		Most likely β-lactamases[e]
		CTX	CAZ				CLOX	CLAV	TEM	SHV	
Group 1	(126)	S	S	Negative	ND[f]	ND	ND	ND	ND	ND	AmpC (WT)
Group 2	(57)	R	R	Negative	8.0–8.9	Yes	Yes	No	ND	ND	AmpC (DM)
Group 3	(29)	S/I/R	I/R	Positive	8.0–8.9	No	Yes	No			AmpC
					8.2	Yes	No	Yes	No	Yes	SHV ESBL
					7.6	Yes	No	Yes	No	Yes	SHV ESBL
					5.6	Yes	No	Yes	Yes	No	TEM ESBL
Wild type[g]	(4)	S	S	Negative	8.3–8.8	No	Yes	No	ND	ND	AmpC (WT)
Derepressed[g]	(5)	R	R	Negative	8.3–8.9	Yes	Yes	No	ND	ND	AmpC (DM)

[a] Group 1 consisted of 51 *E. cloacae,* 18 *E. aerogenes,* 39 *C. freundii,* and 18 *S. marcescens* isolates. Group 2 consisted of 28 *E. cloacae,* 9 *E. aerogenes,* 15 *C. freundii,* and 5 *S. marcescens* isolates. Group 3 consisted of 15 *E. cloacae,* 5 *E. aerogenes,* 7 *C. freundii,* and 2 *S. marcescens* isolates.
[b] Disk diffusion. S, susceptible; I, intermediate; R, resistant.
[c] Hydrolysis of 0.75 μg of CTX per ml used in substrate-based IEF overlay technique (13).
[d] Inhibitors used in IEF overlay technique were clavulanic acid (CLAV) and cloxacillin (CLOX) (13).
[e] WT, wild type; DM, derepressed mutant.
[f] ND, not determined.
[g] Reference 12.

ESBL testing in clinical microbiology laboratories, thus negating the need for ESBL confirmation procedures.

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