Modified double-disc test for detection of extended-spectrum β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

This study evaluates the performance of a modified double-disc test (MDDT) for the detection of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Ninety-six isolates of *E. coli* and 40 *K. pneumoniae* are studied for ESBL production by the National Committee for Clinical Laboratory Standards (NCCLS) combination disc tests and MDDT. A total of 112 (82%) isolates (80 [83%] *E. coli*, 32 [80%] *K. pneumoniae*) were positive for ESBL by MDDT compared to 102 (75%; 72 [75%] *E. coli* and 30 [75%] *K. pneumoniae*) by the NCCLS method. In 10 (7.4%) isolates, ESBLs were detected only by MDDT. Twenty-four (17.6%) isolates were negative for ESBL by both methods. The protocol described in this study provides a more sensitive approach than does the NCCLS method for ESBL detection in *E. coli* and *K. pneumoniae*.

KEY WORDS: Escherichia coli.
Beta-lactamases, extended spectrum.
Klebsiella pneumoniae.

Introduction

Gram-negative bacteria, particularly *Klebsiella pneumoniae* and *Escherichia coli*, that produce extended-spectrum β-lactamases (ESBLs) are reported increasingly worldwide. Production of ESBL and AmpC β-lactamases is the main mechanism for resistance to β-lactam antibiotics among *K. pneumoniae* and *E. coli*.[1,2] The ESBLs are typically plasmid-mediated enzymes that hydrolyse penicillins, third-generation cephalosporins (3GC) and aztreonam.[3,4] They are not active against cephamycins (cefoxitin and cefotetan), but are susceptible to β-lactamase inhibitors (clavulanic acid).[2]

AmpC β-lactamase usually is chromosomally encoded, poorly inhibited by clavulanic acid and can be differentiated from ESBLs by its ability to hydrolyse cephamycins as well as other 3GC.[4,5] Organisms expressing AmpC β-lactamases are not resistant to 3GC unless the enzymes are expressed at high level.[6] Gram-negative organisms that produce both ESBLs and AmpC β-lactamases have been reported worldwide.[7–10] These organisms usually exhibit multidrug resistance that is not always detected in routine susceptibility tests.

Although Clinical Laboratory Standard Institute (CLSI; previously National Committee for Clinical Laboratory Standards [NCCLS]) recommendations exist for ESBL detection and reporting, there are no standard recommendations for detecting ESBL in the presence of AmpC β-lactamases. Therefore, many clinical laboratories may find it impossible to detect ESBL in isolates that simultaneously produce AmpC β-lactamases.

As high-level expression of AmpC β-lactamases may mask recognition of ESBL by the NCCLS method,[5,11] and cefepime, a fourth-generation cephalosporin, is known to be a poor substrate for AmpC β-lactamases,[2] this study included and studied cefepime-clavulanate synergy for ESBL detection in *E. coli* and *K. pneumoniae* in a modified double-disc test.

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Materials and methods

A total of 136 (96 *E. coli*, 40 *K. pneumoniae*) isolates resistant to cefotaxime and/or ceftazidime were studied for ESBL production. The study was conducted at Almana General Hospital, Khobar, Saudi Arabia, over a period of 14 months (October 2004 to November 2005).

Bacterial isolates from various clinical samples were identified by standard techniques and API 20E (bioMérieux, France).[12] The antimicrobial susceptibility of the isolates was determined by the Kirby-Bauer disc diffusion method as recommended by NCCLS.[13] Minimum inhibitory concentration (MIC) for the ESBL-producing bacteria was performed by the agar dilution method. Isolates were tested for ESBL by both standard NCCLS disc method[13] and a modification of the double-disc synergy test.[14]

For the NCCLS method, ceftazidime, cefotaxime and cefpodoxime were used, each with and without clavulanate (ESBL production was indicated by an increase in zone size of ≥5 mm, with and without clavulanic acid). In the MDDT, an amoxicillin-clavulanate disc (AMC, 20 μg-10 μg) was placed in the centre and cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefepime (FEP, 30 μg) and aztreonam (ATM, 30 μg) discs were placed at a distance of 15 mm from the AMC disc and at 90^0 to each other (Fig. 1). This configuration, rather than 20-30 mm, was used as it is

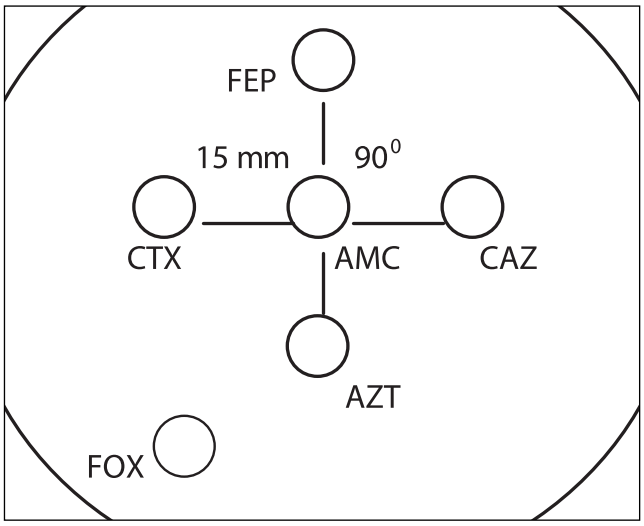

Fig. 1. Antibiotic disc placement in the modified double-disc test. FEP: cefepime (30 μg), CTX: cefotaxime (30 μg), CAZ: ceftazidime (30 μg), AMC: amoxycillin-clavulanate (20 μg/10 μg), AZT: aztreonam (30 μg), FOX: cefoxitin (30 μg).

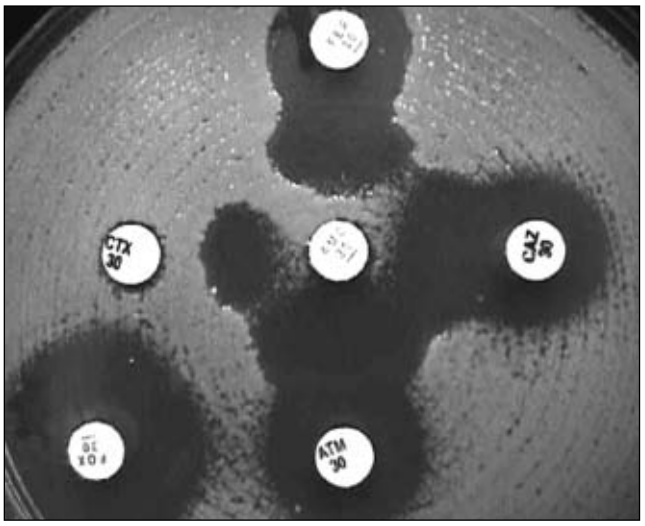

Fig. 2. Modified disc diffusion test. The organism is sensitive to cefoxitin and shows an enhanced zone of inhibition between ceftazidime, cefotaxime, cefepime, aztreonam and amoxycillin-clavulunate (centre disc), indicating a positive result for ESBL.

reported to show greater sensitivity.[15] A cefoxitin disc (FOX, 30 μg) was also placed on the same plate.

Extended-spectrum β-lactamase production was interpreted as positive by a clear extension of the edge of the inhibition zone produced by CAZ, CTX, FEP or ATM towards the AMC disc (Fig. 2). AmpC β-lactamase presence was indicated phenotypically by resistance of the isolate to CTX or CAZ, resistance to both AMC and FOX, and absence of any enhancement of the inhibitory zone towards the clavulanate-containing disc.[16] Control strains of *E. coli* (ATCC 25922, negative control) and *K. pneumoniae* (ATCC 700603, positive control) were used.

Results

Of the 136 isolates, 112 (82%) and 102 (75%) were positive for ESBL by the MDDT and NCCLS methods, respectively (Table 1). Ten (7.4 %) isolates (eight *E. coli*, two *K. pneumoniae*), all of which were positive for ESBL by MDDT (Fig. 3), yielded negative results with the NCCLS disc method. These strains showed a clear extension of the edge of inhibition produced by FEP towards the AMC disc. Figure 4 shows positive ESBL by the NCCLS disc method.

Twenty-four isolates were negative for ESBL by both methods. These isolates were resistant to FOX but susceptible to FEP, and showed no enhancement between any of the cephalosporins or aztreonam and AMC. The phenotypic features shown by these isolates of *E. coli* and *K. pneumoniae* were strongly suggestive of AmpC β-lactamase presence.

Of the 112 ESBL-producing isolates studied, 88 (78.6%) were from in-patients (18 [20%] from the long-term care

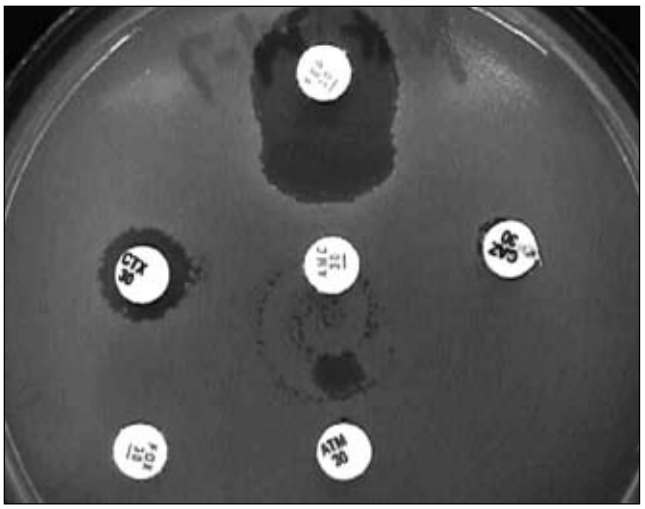

Fig. 3. Modified disc diffusion test. The organism shows resistance to cefoxitin, cefotaxime, ceftazidime and aztreonam and an enhanced zone of inhibition between cefepime (top disc) and amoxycillin-clavulunate (centre disc), indicating a positive result for ESBL and presumptive AmpC β-lactamases.

Table 1. Detection of ESBL among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* by MDDT and NCCLS methods.

	Number of isolates (%)					
	MDDT		NCCLS			
	ESBL+	ESBL–	ESBL+	ESBL–	FOX (S)	FOX (R)
E. coli (*n*=96)	80 (83)	16 (16.7)	72 (75)	24 (25)	72 (75)	24 (25)
K. pneumoniae (*n*=40)	32 (80)	8 (20)	30 (75)	10 (25)	30 (75)	10 (25)
Total (*n*=136)	112 (82)	24 (17.6)	102 (75)	34 (25)	102 (75)	34 (25)

Table 2. Antimicrobial susceptibility of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*.

	Number of isolates (%)						
	FEP	GN	AK	CIP	TZP	IMP	MEM
E. coli (n=80)	8 (10)	26 (32.5)	60 (75)	13 (16)	39 (48.7)	80 (100)	80 (100)
K. pneumoniae (n=32)	13 (40.6)	1 (3)	12 (37.5)	21 (65.5)	11 (34)	32 (100)	32 (100)
Total (n=112)	21 (18.7)	27 (24)	72 (64)	34 (30)	50 (44.6)	112 (100)	112 (100)

FEP: cefepime (30 μg), GN: gentamicin (10 μg), AK: amikacin (30 μg), CIP: ciprofloxacin (5 μg), TZP: piperacillin-tazobactam (110 μg), IMP: imipenem (10 μg), MEM: meropenem (10 μg).

unit, 13 [14.8 %] from the intensive care unit and 57 [64.8%] from other wards) and 24 (21 %) were from out-patients.

All ESBL-producing isolates were susceptible to imipenem and meropenem. Amikacin and piperacillin-tazobactam were active against 64% and 44.6% of the isolates, respectively (Table 2).

Discussion

Clinical isolates of *E. coli* and *K. pneumoniae* often produce multiple β-lactamases.[2] Organisms producing ESBL and AmpC enzymes may be more difficult to identify as ESBL-producing by the standard NCCLS methods. In these bacteria, the co-existing AmpC can hydrolyse the indicator cephalosporin, thereby masking any synergy arising from inhibition of ESBL by clavulanate, and this can prevent recognition of ESBL.[16,17]

In order to overcome this disruption, the present study evaluated the performance of an MDDT based on clavulanate synergy with the 3GC and FEP. The latter, a fourth-generation cephalosporin, is more stable than the 3GCs are to AmpC activity and will demonstrate the synergy arising from inhibition of ESBL by clavulanate in the presence of AmpC enzyme.[18–20]

Failure of the NCCLS disc method to detect the presence of ESBL in AmpC-producing *E. coli* has been reported.[21] The results of the present study indicate that the inclusion of FEP and FOX in the double-disc synergy test increases the sensitivity of the test for ESBL detection. It also demonstrates that ESBLs can be missed by laboratories using the current NCCLS method for ESBL detection in organisms that are also producing AmpC β-lactamases.

Extended-spectrum β-lactamases have been reported in other bacteria including *Enterobacter cloacae* and *Klebsiella oxytoca*. During the study period, 11 clinical isolates of *E. cloacae* were tested that were resistant to CAZ and CTX. Of these 11 isolates, four were positive for ESBL by both MDDT and NCCLS methodologies. Only one isolate was positive for ESBL by the MDDT method and negative by the NCCLS disc method. Another six isolates were negative for ESBL by both methods.

Three isolates of *K. oxytoca* were tested for ESBL production. Only one isolate was positive for ESBL by the MDDT method, with the remaining two being negative for ESBL by both methods. All the isolates negative for ESBL by the MDDT method were also negative by the NCCLS method.

Identification of ESBLs and AmpC β-lactamase-producing isolates would allow clinical microbiologists or infectious disease specialists to formulate policies for empirical antimicrobial therapy, especially in high-risk units where infections due to these organisms are common. It also helps in monitoring the development of antimicrobial resistance and in the implementation of proper hospital infection control measures.

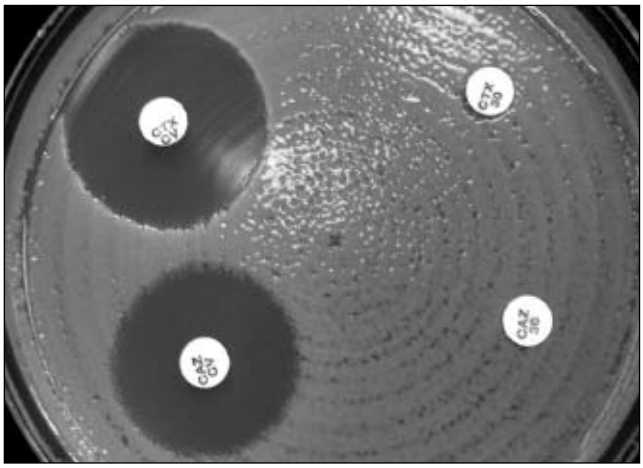

Fig. 4. NCCLS disc confirmation test. ESBL production confirmed by an increase in zone size of more than 5 mm for cefotaxime and ceftazidime (CAZ), with and without clavulanic acid (CV).

Currently, there are no standard phenotypic tests for the simultaneous detection of ESBL and AmpC, and therefore clinical laboratories need to use molecular testing to identify organisms producing both enzymes. However, genotypic methods are beyond the capabilities of many clinical laboratories, and therefore simple procedures for the detection of both enzymes need to be incorporated in routine diagnostic test profiles.

In conclusion, the results of this study indicate that ESBL detection by MDDT is more sensitive than by the standard NCCLS method. Further investigation of the protocol described here to identify clinical isolates harbouring both ESBL and AmpC β-lactamase is warranted as it is simple and can be incorporated in a Gram-negative template for routine antimicrobial susceptibility testing. □

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