Accelerated detection of extended-spectrum β-lactamases in clinical isolates of Enterobacteriaceae

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ABSTRACT

A prospective study is carried out to evaluate the performance of a protocol for the accelerated detection of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae* and other Gram-negative bacteria. A modified double-disc test (MDDT) is incorporated in a Gram-negative template for routine susceptibility testing. The MDDT identified accurately ESBLs in all isolates subsequently confirmed as ESBL-producers by the standard Clinical Laboratory Standards Institute (CLSI) combined disc method. Of 1213 isolates tested, 98 (8%) were positive for ESBLs by MDDT and 95 (7.8%) were positive by the CLSI method. ESBLs were detected in 48 (7.8%) *E. coli*, 21 (8%) *K. pneumoniae*, 12 (5.8%) *Proteus mirabilis*, 13 (18.8%) *Providencia stuartii* and four (6.8%) *Enterobacter cloacae* isolates. Time required for ESBL detection by the MDDT method was one day. The protocol described provides a simple, rapid and low-cost method for early detection of ESBLs in Gram-negative bacteria.

KEY WORDS: AmpC beta-lactamases.
beta-Lactamases.
Gram-negative bacteria.

Introduction

Clinical laboratories play a crucial role in providing guidance in the treatment of microbial infections. This role has become more crucial with the increasing antimicrobial resistance among microbial pathogens and decreasing number of new drugs. Infections due to multidrug-resistant strains of *Escherichia coli*, *Klebsiella pneumoniae* and other Gram-negative bacteria have been causing significant diagnostic and therapeutic problems.[1]

One of the main mechanisms for resistance to β-lactam antibiotics among Gram-negative bacteria is the synthesis of extended-spectrum β-lactamases (ESBLs).[2] Typically, these are plasmid-mediated enzymes capable of inactivating a variety of β-lactam drugs, including extended-spectrum penicillins, third-generation cephalosporins and monobactams. They are not active against cephamycins or carbapenems and are highly susceptible to *in vitro* inhibition by β-lactamases such as clavulanic acid.[3,4]

Organisms producing ESBLs are difficult to identify with routine antimicrobial susceptibility tests. Failure to detect and report ESBL-mediated resistance may lead to treatment failure, especially when cephalosporins are used for therapy.

Delay in the detection and reporting of ESBL producers is associated with prolonged hospital stay, increased morbidity, mortality and healthcare costs.[5,6] Therefore, prompt reporting of ESBL-producing organisms is necessary to ensure that patients receive effective antibiotic therapy, and that appropriate infection control measures are implemented. Furthermore, the early identification of ESBL-producing organisms is particularly important in countries where excessive use of antibiotics is common and in which there is a lack of adequate antimicrobial resistance surveillance.

Extended-spectrum β-lactamase-mediated resistance can be detected by several methods, including the Clinical Laboratory Standards Institute (CLSI) combined disc and the double-disc synergy methods.[7,8] The standard CLSI disc method involves initial screening for ESBL production and then use of confirmatory tests on screen-positive isolates. In the authors' laboratory, the modified double-disc test (MDDT) for ESBL detection in cefotaxime- and/or ceftazidime-resistant isolates of Gram-negative bacteria is being used.

Owing to the high reliability of MDDT for ESBL detection,[9] this study aims to evaluate the performance of the MDDT when incorporated in a routine Gram-negative susceptibility template for simultaneous detection of ESBLs in clinical isolates of Enterobacteriaceae.

Materials and methods

A total of 1213 consecutive (non-repeat) Gram-negative isolates obtained from various clinical specimens sent to the microbiology laboratory at Almana General Hospitals, Khobar and Dammam in the Eastern Province of the Kingdom of Saudi Arabia were included in the study, which was performed during the period January to July 2006. Isolates were identified by the standard techniques and API 20E (bioMérieux, France).[10]

Antimicrobial susceptibility was determined by the disc

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Fig. 1. Modified disc diffusion test. Organism sensitive to cefoxitin showing enhanced zone of inhibition between ceftazidime/cefepime/aztreonam and amoxycillin-clavulanate (central disc) indicating ESBL positivity.

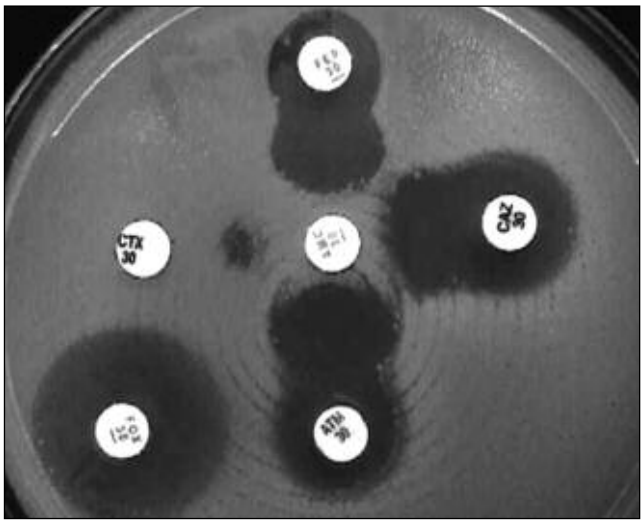
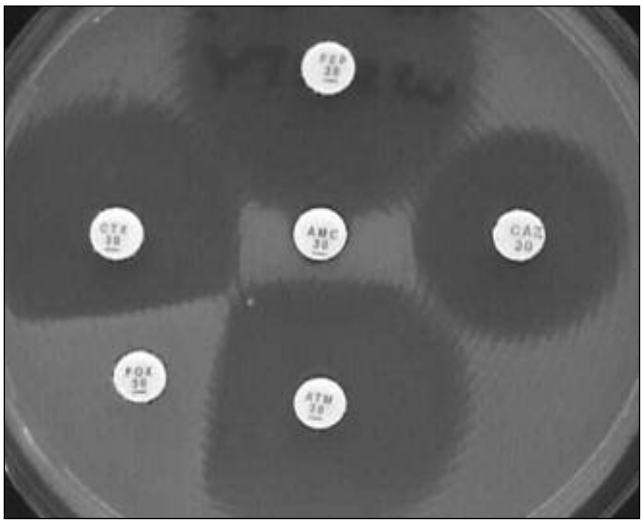


Fig. 2. *Enterobacter cloacae*. Organism showing flattening of cefotaxime (CTX) zone close to cefoxitin (FOX), indicating the presence of inducible AmpC β-lactamase.



diffusion method on Meuller-Hinton medium and results were interpreted according to CLSI criteria.[7] Antibiotics used were amoxycillin (10 µg), cephalexin (30 µg), cefuroxime (30 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), piperacillin (100 µg) and piperacillin/tazobactam (100/10 µg). *E. coli* (ATCC 25922) was used as a control organism for testing the activity or potency of the antibiotic discs used in the study.

The MDDT performed was a modification of the original double-disc test.[8] Amoxicillin-clavulanate (20/10 µg; AMC) disc 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 15 mm from the AMC disc. A cefoxitin disc (FOX, 30 µg) was also placed on the same plate. All discs were obtained from Oxoid, Basingstoke, UK.

Following incubation at 35°C for 18–20 h, a clear extension of the zone of inhibition between any of the CAZ, CTX, FEP or ATM discs and the AMC disc was interpreted as positive for ESBL production (Fig. 1). The standard CLSI combined disc method involving CAZ and CTX with and without the inhibitor clavulanic acid (30 µg; Mast Diagnostics, Merseyside, UK) was used to confirm the presence of ESBL.

Production of ESBL was indicated by an increase in zone size >5 mm, with and without clavulanic acid. Confirmatory CLSI test was performed on both control and clinical strains. For ESBL detection, control strains of *E. coli* (ATCC 25922; negative control) and *K. pneumoniae* (ATCC 700603; positive control) were used.

Table 1. ESBL detection in Gram-negative isolates by the MDDT and CLSI methods.

	No. (%) isolates	No. (%) ESBL-positive isolates	
		MDDT	CLSI
Escherichia coli	618 (50.9)	48 (7.8)	45 (7.3)
Klebsiella pneumoniae	260 (21.4)	21 (8)	21 (8)
Proteus mirabilis	207 (17)	12 (5.8)	12 (5.8)
Providencia stuartii	69 (5.7)	13 (18.8)	13 (18.8)
Enterobacter cloacae	59 (4.9)	4 (6.8)	4 (6.8)
Total	1213	98 (8)	95 (7.8)

MDDT: Modified double-disc test
CLSI: Clinical Laboratory Standards Institute

Results

A total of 1213 Gram-negative isolates (*E. coli* [*n*=618], *K. pneumoniae* [*n*=260], *Proteus mirabilis* [*n*=207], *Providencia stuartii* [*n*=69] and *Enterobacter cloacae* [*n*=59]) were tested. Of the total, 98 (8%) had an ESBL phenotype by the MDDT method, while this phenotype was found in 95 (7.8%) by the standard CLSI combined disc test (Table 1).

Three isolates of *Escherichia coli* were identified as ESBL producers by the MDDT test, but not by the standard CLSI test. Of the 59 isolates of *Enterobacter cloacae* studied, 42 (71%) were susceptible to CTX and CAZ and exhibited D-type inducible AmpC resistance (Fig. 2). The CTX zone of inhibition was D-shaped, with bacterial growth extending towards the CTX disc as a result of induction by FOX.

The ESBL-producing phenotype was found most frequently among *P. stuartii* (18.8%) and *K. pneumoniae* (8%) isolates, with 100% concordance between the results of MDDT and standard CLSI tests.

Carbapenems (imipenem and meropenem) were the most active antibiotics against the ESBL-producing isolates, with 100% activity seen against all ESBL-producing isolates.

Discussion

Hospital laboratories need to detect ESBL-producing strains of the family Enterobacteriaceae adequately and promptly, and especially those that may appear falsely to be

susceptible to broad-spectrum cephalosporins. In the present study, MDDT was used to detect ESBL producers directly. The main modifications to the double-disc synergy test consisted of closer application of the discs (15 mm instead of 30 mm) and the inclusion of cefepime and cefoxitin discs. The use of these modifications, especially the cefepime-clavulanate synergy, which has better discriminatory power between ESBL production and AmpC hyperproduction,[11] has increased the test's sensitivity for ESBL detection.[9]

Using direct testing by MDDT, 8% of the isolates showed an ESBL phenotype, while this phenotype was found in 7.8% by the standard CLSI test. Three isolates of *Escherichia coli* were resistant to cefoxitin and showed synergy only between cefepime and the clavulanate-containing disc. The negative CLSI results obtained for these isolates may have been caused by the presence of AmpC β-lactamases that masked the presence of ESBL.

The protocol used in the present study permits reporting of ESBL-producing organisms on the same day that first-line antimicrobial susceptibility results are read. Thus, it is possible to identify bacterial isolate as ESBL-producing within 24 h, reducing detection time from two days.

This early detection enables the laboratory to advise appropriate antibiotic therapy at an early stage, which is especially important for patients with serious infections not suspected as being caused by these organisms. It also helps the laboratory avoid reporting false susceptibilities to broad-spectrum cephalosporins and allows prompt implementation of appropriate infection control measures.

Early detection of ESBL-producing organisms is particularly important in patients with bloodstream infections (BSIs). Rapid protocols for the accelerated detection of ESBL-producing Gram-negative organisms from positive blood cultures have been reported in several recent studies.[12–14] In these, an ESBL phenotype was determined directly from blood cultures by the combined disc method using cefotaxime and ceftazidime or cefpodoxime with and without clavulanate. In the present study, with the MDDT incorporated in the direct sensitivity test, it was possible to detect an ESBL phenotype in all bloodstream infections caused by ESBL-producing *K. pneumoniae* (n=5) and *E. coli* (n=3).

Extended-spectrum β-lactamase enzymes have been reported in other genera of the family Enterobacteriaceae, such as *Enterobacter cloacae*, *Citrobacter* spp. and *P. stuartii*. Although no criteria for the detection of ESBL production are provided by CLSI for these organisms, excellent correlation has been reported between the standard CLSI phenotypic ESBL assays and the presence of ESBL-encoding genes in non-*Escherichia coli* and non-*Klebsiella* spp. isolates of Enterobacteriaceae.[15,16] High incidences of ESBLs have been reported in *P. stuartii* isolates.[17,18] In the present study, ESBLs were found most frequently in *P. stuartii* isolates.

Other members of the Enterobacteriaceae, such as *Enterobacter cloacae*, occasionally produce high levels of AmpC β-lactamase, due to derepression of the *ampC* locus. Typically, this resistance phenotype emerges during therapy with a third-generation cephalosporin.[19] In the present study, 13 (22%) of the *E. cloacae* isolates showed resistance patterns suggestive of constitutive AmpC β-lactamases (i.e., resistant to ceftazidime and cefotaxime, regardless of the presence of the AmpC-inducing agent cefoxitin).

Another 42 (71%) isolates exhibited inducible AmpC resistance. Strains with inducible AmpC resistance should be considered potentially resistant to third-generation cephalosporins, and these drugs are not recommended for treatment of severe infections caused by these bacteria.[20, 21]

In conclusion, the protocol described in this study is a sensitive, simple and low-cost method for the rapid detection of ESBLs in *E. coli*, *K. pneumoniae* and other Gram-negative bacteria. It also provides an opportunity to apply the technique across microbiology, and laboratories that perform susceptibility testing by disc diffusion can incorporate the MDDT protocol into routine susceptibility testing. □

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