Original Research Article

Evaluation of the NDP Test, a Novel Chromogenic Test for Rapid Detection of Extended Spectrum β-Lactamase Producing Enterobacteriacae

ABSTRACT

Background: The early detection ofextended spectrum β-lactamase(ESBL) producers in clinical microbiology is now of great importance to optimize appropriate therapeutic schemes and to improve the patient outcome.The ESBL NDP (Nordmann/Dortet/Poirel) test has been recently developed for the early detection of ESBL producing organisms.It is based on the biochemical detection of the hydrolysis of the β-lactam ring of cefotaxime (a broad spectrum cephalosporin).

Aims: Of this study was done to evaluate the performance of NDP test in detection of ESBL producing organism directly from urine samples and blood cultures.

Place and Duration of Study: This is a Seven-months Cross sectional study conducted in Internal Medicine and Medical Microbiology & Immunology departments, Benha University,Egypt.

Methodology:A total of one hundred Gram negative bacterial isolates (60 urine isolates and 40 blood isolates) were tested for ESBL production by ESBL NDP test.All isolates were screened phenotypically for ESBL production with disc diffusion method then confirmed using the double disc synergy test (DDST).Characterization of ESBL encoding genes were done by multiplex PCR.

Results: In total,39% were confirmed as ESBL positive using the DDST and PCR. The genetic analysis revealed that CTX-M was the most prevalent gene type (71.8%) followed by SHV genes (35.9%) then TEM genes (20.5%).For the detection of ESBL producers directly from urine samples, NDP test had a sensitivity of 90.5%, specificity of 100%, positive predictive value of 100% and negative predictive value of 95%. NDP test had an excellent performance when performed directly on blood culture, it had sensitivity, specificity, positive predictive value and negative predictive value, all of 100 %.

Conclusion: The NDP test is a rapid, sensitive, and specific test that could be introduced in clinical practice.

Keywords: Enterobacteriacae, ESBL; NDP; DDST.

1.INTRODUCTION

Enterobacteriaceae considered one of the most important causes of both community-acquired and nosocomial infections. The main therapeutic choices for treatment of these organisms are Beta-lactams (mainly extended-spectrum cephalosporins and carbapenems) and fluoroquinolones[1].

One of the most critical emerging resistance developments in Enterobacteriaceae is resistance to broad-spectrum β -lactams, which is particularly related to production of clavulanic-acid that inhibits extended-spectrum β -lactamases (ESBLs). These enzymes are plasmid mediated and responsible for multiple drug resistance as first, second- and third-generation cephalosporins, penicillin and aztreonam. ESBLs have no effect on carbapenems and cephamycins [2].

The majority of ESBLs belong to the TEM-, SHV- and CTX-M-type enzymes and have been reported in Enterobacteriaceae.Class C cephalosporinases (AmpCs) are chromosome encoded but can also be plasmid mediated (pAmpCs)[3].

Laboratory detection of ESBLs is routinely based on phenotypic testing which require a preliminary screening step followed by confirmatory one. Those techniques require a preliminary growth step of 24 to 48 h, this leads to a delay in the initiation of antibiotic therapy[4].

The screening test relies on testing the organism for resistance to an indicator cephalosporin; the most commonly used is cefpodoxime as it is hydrolyzed by three types of enzymes; however, others can also be used as cefotaxime, ceftriaxone ad ceftazidime. To confirm the presence of an ESBL, synergy between the indicator cephalosporin and clavulanic acid needs to be demonstrated (ESBLs are inhibited by clavulanic acid). There are a variety of commercial tools available to do this, including double disc synergy, combination disc method, and specific ESBL –tests[5].

Both screening and confirming the presence of an ESBL producer can be technically difficult, and it is time consuming. This can be a significant clinical problem, as time to appropriate antibiotic is crucial in the management of a septic patient[6].

Molecular detection of ESBLs (PCR and sequencing) remains costly and needs a certain degree of expertise and does not detect all genes encoding enzymes, so are not suitable for routine clinical testing in most laboratories [7]. Moreover, PCR based techniques' results cannot be obtained till at least 48 h after obtaining the clinical samples[8].

Rapid detection of ESBL producing Enterobacteriacea can be done by a novel test,ESBL NDP (Nordmann/Dortet/Poirel). It is a biochemical test that based onchange in colorfrom red to yellow as a result of hydrolysis of β-lactam ring of cephalosporin (cefotaxime) with the release of carboxyl group into the medium, which is reversed by addition of tazobactam in positive test [8].

2. MATERIALS AND METHODS

2.1 Studied Subjects

This study was conducted according to the guidelines of the Research Ethics Committee of Benha Faculty of Medicine and its University Hospitals during the period from June 2016-- to December 2016. This study was done in Internal Medicine and Microbiology and Immunology departments, Benha University. It was carried out on 100Gram negative isolates (60 urine and 40 blood culture isolates). They were collected from 105 adult patients suspected to have urinary tract infection (UTI) and from 98 adult patientswith suspected blood stream infection, respectively, one isolate per patient. A verbal consent was obtained from all patients. Full history taking and clinical examination was done by the physician.

2.2 Urine Sample Collection

105 midstream urine samples were collected in sterile containers. If collected from indwelling catheter the wall at the juncture with the drainage tube was disinfected and sterile syringe was used for the urine specimen collection. Only urine samples recovered from UTI due to Gram-negative bacilli (≥10^4 leukocytes/ml and positive Gram-negative staining) were included in the study. Laboratory diagnosis of UTI in urine samples was based on the presence of 10^5 CFU of microorganisms /ml in urine culture on CLED, then colony identification was done with standard bacteriological and biochemical methods[9].

2.3 Blood Sample Collection

98 blood samples,10 ml each, were collected by standard techniques. Inoculated into aerobic bottles (BD Bactec Plus and Aerobic/F bottles), then incubated in Bactec 9050 fluorescent series instrument for incubation and periodic reading(Becton Dickinson, USA) at 35℃ for up to 5 days. Bottles that gave a positive signal in the BACTEC blood culture system were examined by Gram stain and subjected to identification with standard bacteriological and biochemical methods[9].

Only urine and blood samples positive with Gram negative bacilli were included in the study and subjected to:

1) ESBL-NDP test.
2) Phenotypic detection of ESBLs.
3) Molecular detection of genes encoding ESBLs.

2.4Rapid ESBL-NDP (Nordmann, Dortet, Poirel) test

1.5 ml of infected urine/ 0.5 ml ofEnterobacteriaceae–positive blood culturewas transferred into three Eppendorf tubes(A,B,C). Tubes were centrifuged for 2 min, and then the supernatant was discarded, followed by resuspetion of bacterial pellet in 500 µl distilled water. Tubes were centrifuged again for further 2 min and the supernatant was discarded and the bacterial pellet was resuspended in 100 µl of 20 mMTris-HCl lysis buffer (B-PERII, Bacterial Protein Extraction Reagent, Thermo Scientific, Pierce). 10 ul of concentrated tazobactam solution (40 mg/ml) in the tube C.100 ul of revealing solution containing a pH indicator (phenol red) was added in tube A (control). 100 ul of revealing solution with cefotaxime at 6 mg/ml was added to B and C test tubes. All tubes were incubated at 37℃ for 15 min. Optical read ing of the color change of each tube was used[10,11].The results were interpreted in Table (1)

Table 1.Interpretation of the results

	No antibiotic (tube A)	**Cefotaxime (tubeB)**	**Cefotaxime + tazobactam (tube C)**
No ESBL	Red	Red	Red
ESBL	Red	Orange/Yellow	Red
Cephalosporinase or Cephalosporinase + ESBL	Red	Orange/Yellow	Orange/Yellow
Non interpretable	Yellow	Yellow	Yellow

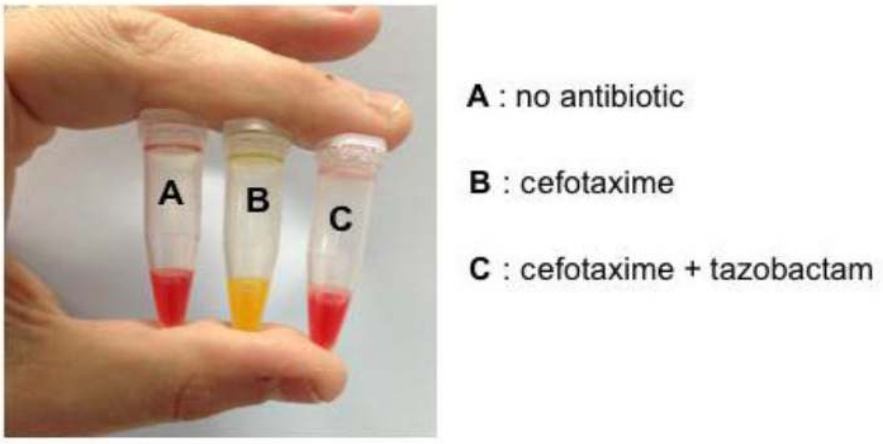

Figure 1. NDP test (ESBL producing Organism)

2.5 Testing for the ESBL Production

2.5.1 Phenotypic screening CLSI method

This was done by antibiotic susceptibility testing (AST) by disc diffusion method (Oxoid,UK) using bacterial colonies grown according to the Clinical Laboratory Standards Institute (CLSI) recommendations [12] (CLSI ,2014). AST results have been interpreted in line with the CLSI breakpoints, as updated in

2014.TheMIC of cefotaxime, ceftazidime, and cefepime have been determined on Muller-Hinton (MH) agar.(*E. coli* ATCC® 25922 was used as quality control strain).
Every strain showed resistance to at least one of the screening antibiotics was picked for ESBL production.

2.5.2 Phenotypic confirmatory test by DDST

We performed double disc synergy test (DDST) (Oxoid,UK)for the confirmation of ESBL production [13].For each strain we used three discs of third generation cephalosporin (cefotaxime 30µg, ceftazidime30µg, cefepime30µg) which were applied 20mm next to a disc with ticarcillin + clavulanic acid that lies in the center of MH agar. A positive result was indicated when the inhibition zones around any of the cephalosporin discs were increased in size more than 5mm in the direction of the disc containing clavulanic acid [12].

2.6 Molecular detection of genes encoding ESBLs:

All positive strains for ESBLs by DDST were subjected to multiplexPCR for characterization of ESBL encoding genes TEM, SHV, CTX-M. The primer sequences for each gene, PCR product sizes and conditions were designed based on published papers are given in Table(2)(Sigma-Aldrich)[14].

Table 2.Primers used for detection of ESBL genes

Target gene	Primer	Primer sequence 5′ to 3′	Size (bp)	PCR conditions
BlaTEM	TEM F	AGT GCT GCC ATA ACC ATG AGT G	431	1-Initial denaturation at 94 ℃ for 5 min. 2-94 ℃ for 20 s,61 ℃ for 30 s and 72 ℃ for 1 min(30 cycles) 3-Final extension of 72 ℃ for 5 min
	TEM R	CTG ACT CCC CGT CGT GTA GAT A		
BlaSHV	SHV F	GAT GAA CGC TTT CCC ATG ATG	214	
	SHV R	CGC TGT TAT CGC TCA TGG TAA		
BlaCTX	CTX F	ATG TGC AGY ACC AGT AAR GT	593	
	CTX R	TGG GTR AAR TAR GTS ACC AGA		

2.6.1DNA extraction:

DNA was extracted from organism by heat lysis. In brief, one pure colony was suspended in 40 µl of sterile distilled water, and the cells were lysed by heating up at 95 ℃ for 5 min. followed by a centrifugation step of the cell suspension. The supernatant that contained the nucleic acid was used for amplification[14].

2.6.2DNA amplification:

25 µl volume in which 12.5 µl of PCR master mix 2× (Thermo scientific), were mixed with 12.5 µl of DNA, primers, and H2O in the following manner; 0.5 µl TEM F, 0.5 µl TEM R, 1 µl of each remaining primers, (SHV, CTX-M) (10 µM/µl), 2.5 µl H2O, and 5 µl of the template DNA. Reactions were performed in a DNA thermal cycler (Applied Biosystems, Foster City, CA, USA)[14].

2.6.3 DNA detection:

All PCR products were electrophoresed in a 2% agarose gel containing 0.3 mg/ml of ethidium bromide. The bands were visualized using UV transilluminator (254nm) & analyzed.

2.7 Statistical Analysis

Data were entered into a database using SPSS 13 for Windows (SPSS Inc., Chicago, IL). Sensitivity: the ability of the test to detect true positive cases and specificity: the ability of the test to detect true negative cases. [15].

3. RESULTS

A total of 60 urine samples and 40 blood samples, positive for Gram-negative bacilli were included in this study. The organisms in urine samples were identified as: 41(68.3%) *E.*coli, 14(23.3%) *Klebsiella* pneumonia, 3 (5%) *Pseudomonas* aeurogenosa, 1 (1.7%) *Enterobacter* spp., and 1 (1.7%) *Proteus* spp. The organisms in blood samples were identified as: 19 (47.5%) *E.*coli, 12 (30%) *Klebsiella* pneumonia, 7 (17.5%) *Enterobacter* spp., and 2 (5%) *Salmonella* typhi (Table 3).

Among those 100 isolates, 43 (43%) were found ESBL positive following preliminary screening, from which 39 (39%) (21 from urine samples and 18 from blood samples) were subsequently confirmed as ESBL positive by DDST and PCR.

The frequency of ESBL production among the urine isolates was (36.5%) (15/41) of*E*. coliand (42.9%) (6/14) of*Klebsiella* pneumonia. However, that in blood isolates was as follows: (52.6%) (10/19) of *E*. coli,(41.7%) (5/12) of *Klebsiella* pneumoniaand (42.9%) (3/7) of *Enterobacter*spp.(Table 3).

Table 3. Prevalence of ESBL production among Gram negative isolates.

Urine (n = 60)			**Blood (n = 40)**		
Organism	**n (%)**	**ESBL producer n (%)**	**Organism**	**n (%)**	**ESBL producer n (%)**
*E.*coli	41(68.3%)	15(36.5%)	*E.*coli	19(47.5%)	10(52.6%)
*K.*pneumoniae	14(23.3%)	6 (42.9%)	*K.*pneumoniae	12(30%)	5 (41.7%)
*P.*aeuroginosa	3 (5%)	0 (0.0%)	*Enterobacter* spp.	7 (17.5%)	3 (42.9%)
Enterobacter spp.	1 (1.7%)	0 (0.0%)	*Salmonella* typhi	2 (5%)	0 (0.0%)
Proteus spp.	1 (1.7%)	0 (0.0%)			
Total	**60**	**21**	**Total**	**40**	**18**

ESBL producing isolates were classified according to their susceptibility to cefotaxime (the substrate used in the ESBL NDP test) into: cefotaxime resistant isolates (n = 37) and cefotaxime sensitive isolates (n = 2). Both cefotaxime sensitive isolates were originated from urine samples.

The result of the molecular analysis revealed that CTX-M was the most prevalent gene type, it was present in 71.8% (28/39)of the ESBL-producing isolates followed by SHV genes 35.9% (14/39) then TEM genes 20.5% (8/39). There were multiple occurrences of genes in some of the isolates (Table 4).

Table 4. Distribution of ESBL genes among the study isolates.

Gene type	***E.*coli (n = 25)**	***K.*pneumoniae(n = 11)**	***Enterobacter* spp. (n = 3)**	**Total (n = 39)**
CTX-M	14	3	2	19
SHV	2	5	0	7
TEM	3	0	1	4
CTX-M + SHV	3	2	0	5

CTX-M + TEM	2	0	0	2
SHV + TEM	0	0	0	0
CTX-M + SHV + TEM	1	1	0	2

3.1Results of the ESBL NDP test:

In total, 37 (37%) of the 100 urine and blood samples were found to be NDP positive, 62 (62%) were negative and one (1%) gave uninterpretable results.

3.1.1In urine samples:

All urine samples (n = 60) gave interpretable results, 19 (36.7%) of them were found to be NDP positive and 41 (63.3%) were negative. All NDP positive isolates were confirmed as cefotaxime resistant ESBL producers, however, all NDP negative isolates were confirmed as non-ESBL producers except for two isolates which were confirmed as cefotaxime sensitive ESBL producers (Table 5). For the detection of ESBL producers directly from urine samples, NDP test had a sensitivity of 90.5%, specificity of 100%, positive predictive value (PPV) of 100% and negative predictive value (NPV) of 95%.

3.1.2 In blood samples:

18/40 (45%) of the blood samples were found to be NDP positive, 21/40 (52.5%) were negative and one isolate (2.5%) (corresponded to a non-ESBL producer) gave uninterpretable result. As expected, all NDP positive isolates were confirmed as ESBL producers and all NDP negative isolates were confirmed as non-ESBL producers (Table 5). With excluding an isolate with uninterpretable result or considering it negative, NDP test had an excellent performance when performed directly on blood culture, it had sensitivity, specificity, PPV and NPV, all of 100 %.

Table 5. Result of NDP test among the study isolates.

Urine (n = 60)						**Blood (n = 40)**					
ESBL (n = 21)			**Non-ESBL (n = 39)**			**ESBL (n = 18)**			**Non-ESBL (n = 22)**		
NDP +ve	NDP -ve	NIP	NDP +ve	NDP -ve	NIP	NDP +ve	NDP -ve	NIP	NDP +ve	NDP -ve	NIP
19	2	0	0	39	0	18	0	0	0	21	1

NIP, Non-interpretable

4. DISCUSSION

The emergence of plasmid mediatedextended spectrum β-lactamases(ESBLs) among the members of Enterobacteriaceae have increased worldwide.It is recognized that Egypt has an extremely high rate of ESBL producers, with up to 70% of isolates producing the enzyme.One survey compared data from Egypt, Lebanon, Saudi Arabia, and South Africa, and Egypt was found to have the highest rates of ESBLs.Possibly, this high prevalence is related to the less controlled use of antibiotics in Egypt, where many drugs are still available over the counter[6].

In this study, the overall rate of ESBL was 39%, andthe maximum ESBL production was seen among the isolates of*E.*coli(64.1%) and*Klebsiella*pneumoniae (28.2%). The reasons for high ESBL in these species

might be due to the fact that these organisms tend to cause nosocomial infection more than others hence it has more chance to acquire multi drug resistance plasmids.

The high ESBL rate detected in this study is in agreement with that recorded by another study from Egypt,Bouchillon et al conducted the PEARLS study in 2001–2002, and found that 38.5% of Enterobacteriaceae isolates produced an ESBL[16].In 2009, a higher rate of ESBL prevalence (64.7%) was recorded by Ahmed et al among strains of Enterobacteriacaeisolated from patients in the intensive care unit of a university hospital[17].Also,Abdallah et al, in their study, found that 48.93%of the tested clinical strains of Enterobacteriaceae isolated from blood of Egyptian patients with suspected blood stream infection were ESBL positive[18].However, a lower ESBL prevalence rate (16%) was found among 120 isolates collected between May 2007 and August 2008 at the Theodor Bilharz Research Institute, Cairo, Egypt[19].

The results obtained in this study showed that CTX-M type was the most prevalent β-lactamase-encoding gene. It was detected in almost 71.8% of the ESBL-producing isolates.

These findings agree with other studies from around the world that show that ESBL genes of the CTX-M are dominant [20,21].Also, many studies reported that CTX-M was the most prevalent ESBL gene type in Egypt[18,19,22]**.** In contrast to our findings, Ahmed et al, reported that TEM was the most frequent β-lactamase-encoding gene[17].

Extended-spectrum β-lactamases are an increasing healthcare problem and their rapid detection is therefore crucial in order to prevent their dissemination and to optimize antimicrobial treatment and patient care.

The ESBL NDP test has been developed recently for rapid identification of ESBL [8]. The ESBL NDP test has previously been validated using cultured bacteria and the results are obtained within less than 1 h [8]. Then the protocol of the ESBL NDP test has been modified and led to a shorter period of detection, which was reduced from 60 min to 15 min[10].

In this study, we evaluated the ESBL NDP test directly from blood cultures and directly from urine samples and the overall results was encouraging and promising. Among the all 100 tested samples, we recorded only one (1%) non-interpretable one, it was a blood sample which was included in non-ESBL producing isolates. This result agrees with that reported by Dortet et al.[10] that the rate of non-interpretable results with the ESBL NDP test is very low (1.3%), making this test adequate for routine use.

When this test performed directly on blood culture, the result was excellent, it gave 100% sensitivity, specificity, PPV and NPV. This result come in agreement with that of Nordman et al. [8], they compared the results of this test when performed on colonies cultured on selective media and when performed on spiked blood culture and they found that the overall sensitivity of the ESBL NDP test was even higher (reaching 100%) using the blood culture protocol. They explained this result by the increased inoculum recovered from blood culture experiments compared to those recovered during pure culture experiments. In 2015, Dortet et al. [11]. Evaluatedthe test prospectively in clinical settings directly from blood cultures and also recorded 100 % sensitivity, specificity, PPV and NPV.

When this test performed directly on urine samples, the overall sensitivity, specificity, PPV and NPV were 90.5%, 100%, 100% and 95%, respectively. The only two NDP negative strains isolated from urine samples werecefotaxime susceptible ESBL producers, however, it was effectively detected all cefotaxime resistant isolates. Also, the single negative result recorded by Dortet et al.[10] was a TEM-24 cefotaxime susceptible ESBL producer.

NDP test, as observed in our work, is effective in detecting ESBL producers of all types of genes tested in this work. The only two negative isolates showed coexistence of multiple genes, one of them carried CTM-X&SHV and the other carried the three tested genes. This result for somewhat disagreed with that

recorded by Nordman et al.[8],they reported that NDP test is particularly effective for detecting the CTX-M producers and there is lack of detection of several ESBL producers, in particular of the TEM and SHV series. They explained this result due to weak hydrolysis of cefotaxime and from low-level production of the ESBL related to low MIC values of cefotaxime.

When this test compared with other rapid tests used for detection of ESBL (B-Lacta and Rapid ESBL Screen tests, in a work done by Poirel et al. [4], it showed the greatest performance and the authors concluded that this test will be an alternative to molecular techniques.

NDP test is rapid assay,its implementation directly on blood culture and urine samples can obtain results in 20-30 minutes, and hence, can significantly gain time (at least 24 h) compared to standard phenotypic techniques. This rapid and accurate detection of ESBL producing organisms could facilitate implementation of a rapid therapeutic scheme and hence significantly improve the outcome of infected patients.

In addition, the ESBL NDP test is inexpensive technique when compared to molecular techniques and this may therefore find an excellent applicationin developingcountriesand countries where a high incidence of ESBL producers occurs.Also, it is easy to perform with no special technical experience required,makingit easily be integrated in the laboratory workflow.

5. CONCLUSION

NDP test is a rapid, inexpensive,sensitive and specific test for detection of ESBL producers and could be introduced in clinical practice.

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