

## Full Length Research Paper

## Detection of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* using the ESBL NDP test and flow cytometric assay in comparison to the standard disc diffusion

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This study was undertaken to evaluate the comparison among three different assays: extended-spectrum beta-lactamases (ESBL) Nordmann/ Dortet/ Poirel (NDP) test, flow cytometric assay and disc diffusion method for the detection of ESBL production. Sixty clinical isolates of *Klebsiella pneumoniae* were isolated from patients' clinical samples admitted to Suez-Canal University Hospital, Ismailia Governorate. The percentages of ESBLs producing *Klebsiella pneumoniae* ranged from 70 to 80% by ESBL NDP and flow cytometric assays, respectively in comparison to 76.6% by disc diffusion method. The sensitivity and specificity of the three assays were evaluated and the sensitivity by ESBL NDP and disc diffusion method was 100%, while by the flow cytometric assay, it was 91.3%. The specificity of disc diffusion method in detection of ESBLs was 100%, followed by the ESBL NDP test (85.7%) and flow cytometric assay (77.8%). Kappa testing showed perfect agreement between the ESBL NDP test and disc diffusion method ( $\kappa=0.9$ ), while flow cytometric assay showed substantial agreement ( $\kappa=0.7$ ). The ESBL NDP test offers an applicable tool for rapid detection of ESBL-production. Although, flow cytometric assay is a promising method that might be used in the clinical microbiology laboratory but there is a need for the experienced personnel along with the device.

**Key words:** Extended-spectrum beta-lactamases (ESBLs), ESBL NDP test, flow cytometry.

### INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) produced by Gram-negative bacteria are considered one of the largest and rapidly evolving group of plasmid-mediated enzymes that confer resistance to oxyimino-cephalosporins and monobactams (Pitout, 2010).

*Escherichia coli* and *Klebsiella pneumoniae*, being the major source of community- and hospital-acquired infections are mostly ESBL producers (Pitout and Laupland, 2008).

ESBL recognition has an important clinical impact as

inappropriate treatment can lead to therapeutic failures and consequently to adverse clinical outcomes (Schwaber and Carmeli, 2007). A variety of ESBLs have been reported in *Enterobacteriaceae*, being mostly of the CTX-M-, TEM- and SHV-types (Bush and Jacoby, 2010; Poirel et al., 2012). ESBL detection is necessary to screen patients, improve hospital infection control practices and to curb inappropriate antibiotic use that prolonged the efficacy of the currently available antibiotics (Schwaber et al., 2006; Zahar et al., 2009).

Current techniques for detecting ESBL producers are based on the determination of susceptibility to expanded-spectrum cephalosporins followed by the inhibition of the ESBL activity, mostly by clavulanic acid or tazobactam (Drieux et al., 2008). Sensitivities and specificities of the double disk test and of the E-test proposed for that purpose are good, ranging from 80 to 95% (Gazin et al., 2012). The automated methods used in the detection of ESBL producing organisms had a much higher sensitivity (80 to 99%) than specificity (50 to 80%). However, those tests require overnight growths consuming 24-48 h before ESBL production is detected with a subsequent delay in the initiation of appropriate antibiotic therapy (Schwaber et al., 2006; Drieux et al., 2008; Gazin et al., 2012).

Molecular detection of ESBL genes (PCR and sequencing) is an interesting alternative but remains costly and requires a certain degree of expertise (Drieux et al., 2008; Gazin et al., 2012) since recently, real time PCR and DNA microarray (Check-Points) are commercially available to detect ESBL gene variants (Cuzon et al., 2012). However, those PCR-based techniques require isolation of bacteria from clinical samples prior to susceptibility testing and phenotypic identifications and hence; those results can be obtained at least 48 h after obtaining the clinical samples. Also, they are usually not performed in a routine laboratory but restricted to epidemiological purposes. Therefore a simple and efficient technique for ESBL producers is required (Nordmann et al., 2012).

The ESBL NDP test is a novel test, based on the hydrolysis of the  $\beta$ -lactam ring of a cephalosporin (cefotaxime), which generates a carboxyl group, by acidifying a culture medium. It uses 96-well microtiter plates or a single tube and the acidity resulted from this hydrolysis is identified by the color change using a pH indicator (red phenol) while, inhibition of ESBL activity is evidenced by adding tazobactam in a complementary well (Cuzon et al., 2012).

A rapid, powerful high-throughput technology allowing analysis of several thousand cells per second and providing quantitative and statistically significant data is the flow cytometry (FC) (Shapiro, 2001). Bacterial cells

are incubated with cephalosporins (ceftazidime or cefotaxime) in the presence and absence of clavulanic acid; subsequently, cells are stained with the fluorescent dye Bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)] which is able to diffuse across depolarized membranes. Susceptible isolates display increased fluorescence after 1 h of incubation; conversely, the increase of the depolarized population was only observed after incubation with clavulanic acid associated with ceftazidime or cefotaxime in ESBL producers (Ramos et al., 2012).

In the present study, two new methods (a flow cytometric assay and the ESBL NDP test) were assessed for detection of ESBLs in clinical isolates of *Klebsiella pneumoniae* in comparison with the standard disc diffusion method.

## MATERIALS AND METHODS

### Bacterial strains

A total of 60 clinical isolates of *K. pneumoniae* were isolated from patients (24 males and 36 females) with different clinical infections (12 sputum, 26 urine, 12 pus and 10 blood samples) admitted to Suez-Canal University Hospital, Ismailia Governorate from January to August 2014. The samples were collected from various clinical origins. Blood samples were inoculated into blood culture bottles (Egyptian Diagnostic Media, Egypt) then incubated at 37°C for 7-14 days. Subcultures were done every 48 h on blood agar and MacConkey's agar (Oxoid, UK) plates. Other samples were cultured on nutrient agar (Oxoid, UK) blood agar and MacConkey's agar. Gram negative bacilli giving non-lactose fermenting colonies on MacConkey's agar were taken for biochemical test including mannitol motility, triple sugar ion, indole, citrate, MR, VP and carbohydrate utilization tests for identification (Birgul, 2010). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as ESBL- positive and negative, respectively (CLSI, 2014). All isolates were kept in soft agar at -20°C till the time for ESBL detection.

### Antimicrobial drugs and ESBL phenotypic detection

For the disc diffusion method, antibiotic discs of ceftazidime (CAZ, 30  $\mu$ g), cefotaxime (CTX, 30  $\mu$ g), Cefotaxime- clavulanic acid (30/10  $\mu$ g) (CTC 40  $\mu$ g) and Ceftazidime- clavulanic acid (30/10  $\mu$ g) (CZC 40  $\mu$ g) were purchased from Bioanalyse Chemical Co Ltd, Turkey. Cefotaxime sodium salt, tazobactam (TZB) and clavulanic acid (CLA) were purchased from Sigma-Aldrich, Saint-Quentin-Fallavier, France for the ESBL NDP test. For flow cytometric assay, bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)], a fluorescent probe that binds to membranes and to intracellular proteins of depolarized cells, was purchased from Invitrogen/Life technologies, Carlsbad, USA; a stock solution (1 mg/ml) was prepared in dimethyl sulphoxide (DMSO).

### The disc diffusion method

Stored isolates were subcultured on MacConkey's agar and the

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**Figure 1.** Representative results of the ESBL NDP test. Strains 1 and 2 are negative and positive controls, respectively; strains 3, 5,6,7,8 and 11 are ESBL producers; strains 4,9,10 and 12 are non-ESBL producers.

pure isolated colonies of identified bacteria was adjusted to 0.5 McFarland turbidity standards in 0.85% saline and lawn culture was spread using sterile swabs on Muller Hinton Agar media (Hi-media). All the strains were screened for ESBL production using CTX (30 µg) and CAZ (30 µg). Strains showing zone of inhibition of  $\leq 27$  mm for CTX and  $\leq 22$  mm for CAZ were selected for ESBL combined disc conformation test. Combined discs of CTC (40 µg) and CZC (40 µg) were used in the confirmation test according to the CLSI M2-A10 protocol (CLSI, 2009).

#### **The ESBL NDP (Nordmann/ Dortet/ Poirel) test**

Strains were isolated on MacConkey's agar and incubated at 37°C for 24 h before performing the NDP rapid ESBL test as described by Nordmann et al. (2012). Briefly, one calibrated loop inoculum (10 µl) of the tested strain was resuspended in 150 µl of 20 mM Tris-HCl lysis buffer in eppendorf tubes containing microbeads. Then, microbead tubes were vortexed for 30 min at room temperature for the mechanical lysis of bacteria. After centrifugation, 30 µl of the supernatant was mixed in a well of a 96-well tray with 100 µl of a 1 ml solution made of 3 mg of purified cefotaxime sodium salt in a pH 7.8 phenol red solution. The pH value was then adjusted to a 7.8 value by the addition of drops from 1 N NaOH solution. Mixture of the phenol red solution and the enzymatic suspension being tested was incubated at 37°C for 30 min. Similarly, culture extracts were analyzed in wells containing cefotaxime and tazobactam (4 mg/ml). A test was considered as positive when the well containing cefotaxime alone turned from red to yellow/orange and the well containing cefotaxime supplemented with tazobactam remained red (ESBL producer).

#### **Flow cytometric analysis**

Bacterial isolates from fresh agar plates were inoculated in trypticase soy broth and incubated at 37°C with shaking until the log phase was reached (about 1 h and 15 min). Subsequently, a suspension containing  $5 \times 10^6$  cells/ml in fresh medium was prepared and the bacterial cells were exposed either to 4 mg/L of CTX, or 16 mg/L of CAZ, alone or with 4 mg/L of CLA, for 60 and 120 min. In parallel, after incubation, the cells were centrifuged and washed in PBS. The dye DiBAC4 (3) was added in a concentration of 1 µg/ml for 30 min, at room temperature and protected from light.

The flow cytometric assay was used according to Ramos et al. (2012). It was performed on a FACSCalibur flow cytometer (BD, Sparks, USA). Nearly, 10,000–30,000 events of each sample were measured with the Software Cell Quest. The acquisition settings were defined using non-treated, non-stained cells (autofluorescence) and after adjusting the photomultiplier tubes' voltage to the first logarithmic (log) decade. The fluorescence intensity at 530/30 nm (FL1) was registered after incubation with antimicrobials and staining with 1 µg/ml DiBAC4 (3).

#### **Statistical analysis**

Sensitivity, specificity, positive and negative predictive values were assessed for the ESBL NDP test and the flow cytometric assay considering the standard disc diffusion method as a gold standard. The kappa values were calculated to evaluate the agreement between each of the ESBL NDP test and the flow cytometric assay and the disc diffusion method (Viera and Garrett, 2005).

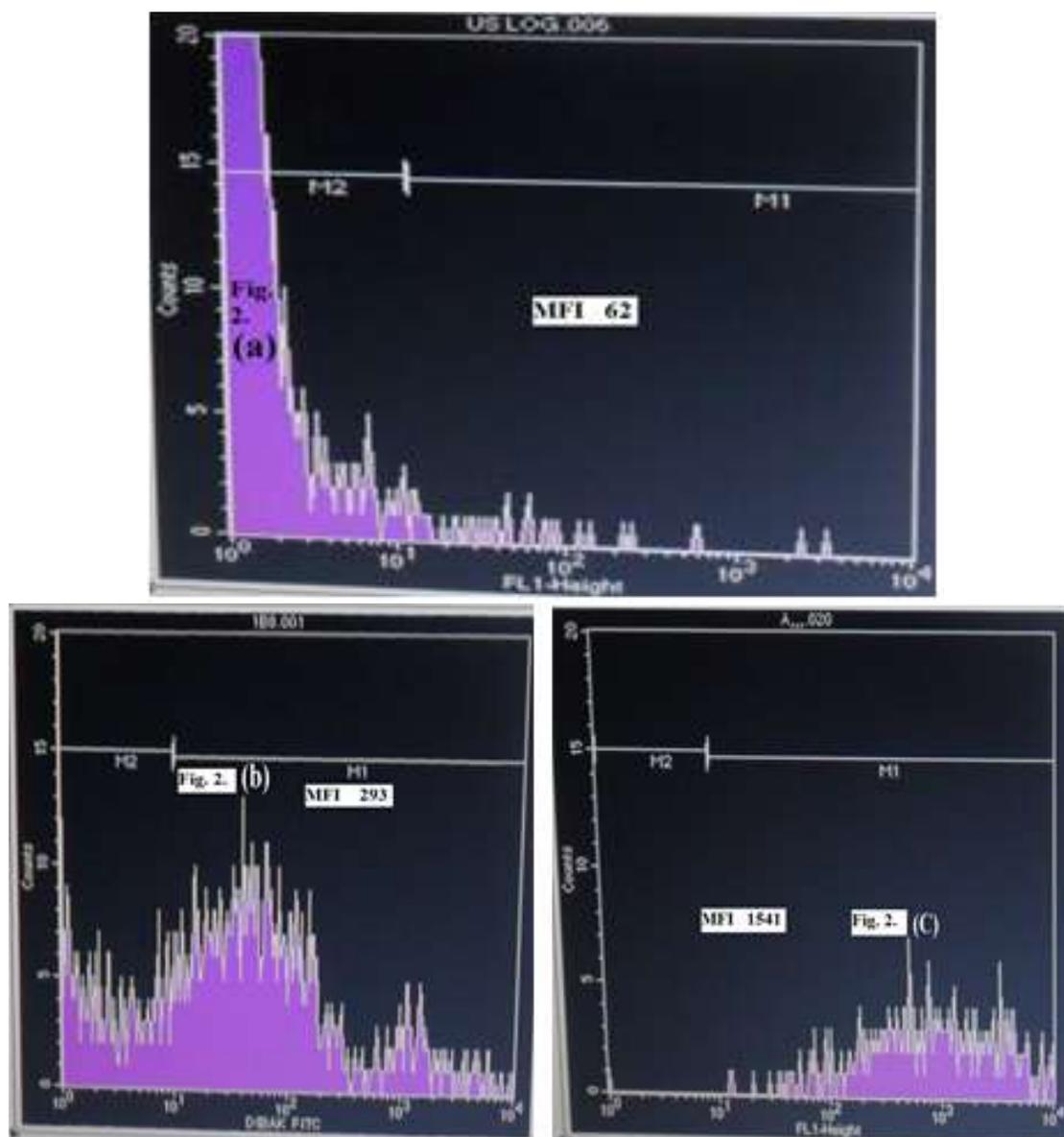
## **RESULTS**

The disc diffusion method had classified the 60 tested strains into 46 (76.6%) ESBL producers and 14 (23.3%) non-ESBL producers. Using the disc diffusion method, an ESBL producer isolates showed resistance to CTX and CAZ then the susceptibility increased ( $\geq 5$  mm increase in zone diameter) to combined discs CTC and CZC while non-ESBL producer isolates were resistant to CTX and CAZ with no increase in the susceptibility to combined discs CTC and CZC.

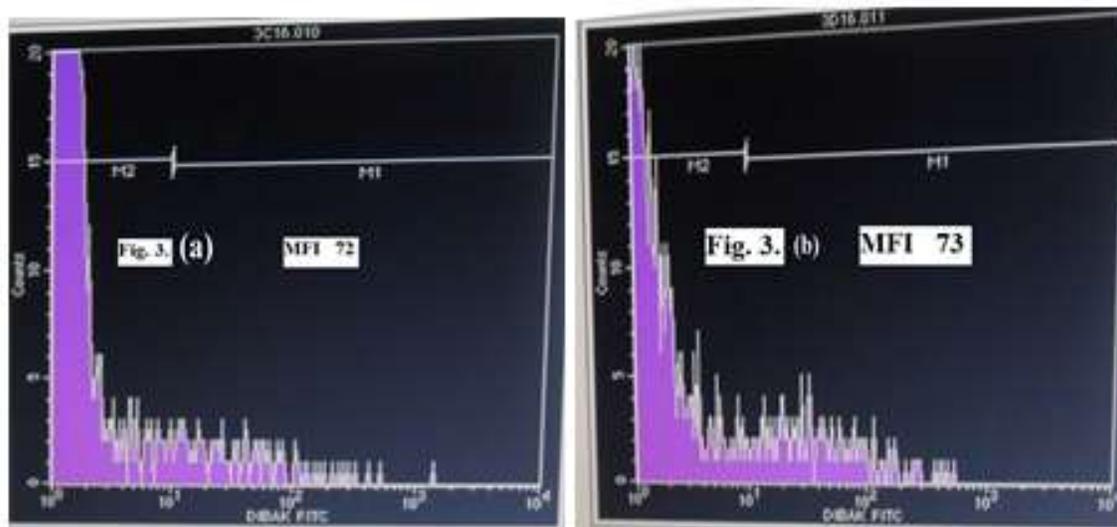
Using the ESBL NDP test, 80% (n= 48) of the tested isolates produced ESBLs as the color of the wells turned from red to yellow in presence of cefotaxime and remained red when tazobactam was added (Figure 1) and 20% (n=12) tested negative for ESBL production. The sensitivity and specificity of the test were 100 and 85.7%, respectively in comparison with the standard disc diffusion method whereas the positive and negative

**Table 1.** Results of the disc diffusion method, the ESBL NDP test and the flow cytometric assay for detection of ESBLs in clinical isolates of *Klebsiella pneumoniae*.

Test result	The disc diffusion method	The ESBL NDP test	Flow cytometric assay
ESBL producers	46	48	42
Non- ESBL producers	14	12	18
Total	60	60	60
Sensitivity	100%	100%	91.3%
Specificity	100%	85.7%	77.8%
Positive predictive value		95.8%	91.3%
Negative predictive value		100%	77.8%



**Figure 2.** Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of (a) non-treated and non-stained cells (autofluorescence) with mean fluorescence intensity (MFI) of 62 (b) an example of ESBL producer isolate after treatment with CTX (4 mg/L) for 60 min; the MFI was 293, and (c) after treatment with CTX (4 mg/L) and CLA (4 mg/L) for 60 min: the MFI was 1541.



**Figure 3.** Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of an example of a non-ESBL producer isolate. (a) After treatment with CTX (4 mg/L) for 60 min; the MFI was 72. b) After treatment with CTX (4 mg/L) and CLA (4 mg/L) for 60 min: the MFI was 73.

predictive values of this test were 95.8 and 100%, respectively. Kappa testing showed an almost perfect agreement between the ESBL NDP test and disc diffusion method in detecting ESBLs ( $\kappa=0.9$ ).

Out of the 60 tested isolates, 42 (70%) and 18 (30%) were ESBL and non-ESBL producers, respectively with the flow cytometric assay method. The sensitivity and specificity of the test were 91.3 and 77.8% whereas the positive and negative predictive values of this test were 91.3 and 77.8%, respectively in comparison with the standard disc diffusion method (Table 1). The intrinsic autofluorescence signal of bacterial cells was detected at the first decade of the logarithmic scale [the mean fluorescence intensity (MFI) was 62]. This corresponds to very low fluorescence intensity without interference with the assessment of membrane depolarization using DIBAC4 (3) as a voltage sensor probe (Figure 2a). Higher intensity of green fluorescence (530/30 nm - FL1) was obtained with dead cells compared with viable cells; consequently, two distinct regions were defined, respectively, for depolarized and polarized cells after staining with DIBAC4 (3). Considering the ESBL-positive clinical isolates, the MFI was 293 after treatment with CTX for 60 min, and then drastically increased to 1541 following simultaneous incubation with both CTX and CLA for 60 min (Figure 2b, c). For non-ESBL producer isolates, the MFI was 72 after treatment with CTX (4 mg/L) for 60 min and remained around value 73 after treatment with both CTX and CLA for 60 min (Figure 3a,b).

For evaluation of agreement between the flow cytometric assay and disc diffusion method, Kappa testing showed substantial agreement between both tests

( $\kappa=0.7$ ).

## DISCUSSION

ESBLs are the main cause of resistance to beta-lactam antibiotics which are among the safest and most frequently prescribed antimicrobial agents all over the world. As their occurrence has been increasing, it becomes essential to evaluate their occurrence in *E. coli* and *K. pneumoniae* which are mostly ESBL producers (Pitout and Laupland, 2008; Sahu et al., 2011).

The incidence of ESBL-producing *K. pneumoniae* varies from country to another depending upon various factors, like antibiotic policy, the carriage rate among hospital personnel, and the type of disinfection used especially in the ICU (Sarojamma and Ramakrishna, 2011). It is recognized that Egypt has an extremely high rate of ESBL producers, with up to 70% of isolates producing the enzyme (Borg et al., 2006). In the present study, 76.6% ( $n=46$ ) of the 60 tested strains were ESBL producers and 23.3% ( $n=14$ ) were non-ESBL producers. This could be attributed to the empirical usage of 3rd generation cephalosporins in treatment of nosocomial infections in our hospitals.

Although molecular methods brought speed and accuracy, they are costly and not suitable for low income developing countries (Gazin et al., 2012). In this work, we assessed two phenotypic methods; the ESBL NDP test and flow cytometric assay for detection of ESBLs in *K. pneumoniae* clinical isolates in comparison with the standard disc diffusion method. The ESBL NDP test was able to detect all ESBL-producing isolates that hydrolyze

cefotaxime (color change from red to yellow in the first well), while the second well that contained tazobactam remained red (inhibition of hydrolysis), thus corresponding to a positive test. The sensitivity and positive predicative value of the test were 100 and 95.8%, respectively. This result was higher than that of Nordmann et al. (2012) who evaluated the ESBL NDP test retrospectively on a collection of 255 strains (from various clinical and geographical origins and previously characterized at the molecular level). In their published study, the sensitivity of the test was 92.6%. Also, our results are higher than those of Dortet et al. (2014) who applied the ESBL NDP test on 500 ESBL producing Enterobacteriaceae recovered from urine samples. They reported that the sensitivity of the ESBL NDP test was 98% while the positive predicative value was 98% which is higher than ours. The discrepancy of the results may be attributed to the different geographical origins and the large number of tested isolates in comparison with our study. Two false positive isolates were detected by the ESBL NDP test as some isolates could contain combined ESBL and AmpC-overproducing enzymes giving a positive result, if the corresponding AmpC hydrolyses cefotaxime at high level.

The specificity and the negative predictive value of the ESBL NDP test in our study were 85.7% and 100%, respectively. These results are lower than those of Nordmann et al. (2012) and Dortet et al. (2014) whereas, it was 100% in the first study and 99.8% in the second one. This could be explained by the inability of the test in detecting non-CTX-M ESBL producers and strains which had MIC values of cefotaxime lower than the resistance breakpoint for that molecule (>8 µg/ml).

Our results show an almost perfect agreement between the ESBL NDP test and disc diffusion method in detecting ESBLs ( $\kappa=0.9$ ) which agrees with those of Dortet et al. (2014) who observed a perfect correlation between cefotaxime resistance and positivity of the ESBL NDP test.

Compared to the standard disc diffusion method, flow cytometric assay yielded a sensitivity of (91.3%) while the specificity was 77.8%. It correctly detected 42 isolates out of the 46 ESBL positive isolates previously catalogued by the standard disc diffusion method. Only 4 strains tested false negative result which might be obtained whenever complex mutant or rare ESBL types are present as isolates expressing these enzymes confer resistance to cephalosporins but are partially inhibited or not inhibited by CLA acid, respectively (Canton et al., 2008; Drawz and Bonomo, 2010).

Our results are in concordance with those of Ramos et al. (2012) who tested 20 ESBL-negative and 41 ESBL-positive isolates phenotypically catalogued by the standard disc diffusion method and molecular typing. In their study flow cytometric analysis correctly detected all the 41 ESBL-positive isolates. It showed an excellent correlation either with phenotypic analysis or molecular typing however, in our study flow cytometric analysis

showed substantial agreement with the standard disc diffusion method ( $\kappa=0.7$ ).

The ESBL NDP test offers a simple and rapid test with an almost perfect agreement with the standard disc diffusion method in detecting ESBLs which could significantly help in guiding first-line antibiotic therapy and improve the outcome of infected patients. Flow cytometric assay is a promising method that might be used in the clinical microbiology laboratory provided that the availability of the device and a trained personnel. Nonetheless, the standard method remains the best one because of its low price for the lab and the patient.

## Conflict of Interests

The authors have not declared any conflict of interest.

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