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RESEARCH ARTICLE

RAPID DETECTION OF EXTENDED SPECTRUM B – LACTAMASES (ESBL) AND THEIR *CTX-M* GENETIC CHARACTERIZATION.

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Abstract

Background: The recently described ESBL-NDP (Nordmann Dortet Poirel) test is a promising rapid and inexpensive phenotypic test used to recognize extended-spectrum-β-lactamases (*ESBLs*). *Bla* $_{CTX-M}$ gene is recently replacing *bla* $_{SHV}$ and *bla* $_{TEM}$ genotypes as the prevalent ESBL resistance genes and their detection may aid in infection control efforts and epidemiology of resistance.

Aim: The current study aimed to detect the burden of *ESBLs* by the rapid ESBL-NDP test, and to examine the prevalence of *CTX-M* gene among *Enterobacteracea* in Egyptian patients.

Material and Methods: One hundred forty seven Gram negative *Enterobacteracea* isolates were screened for *ESBL* production with disc diffusion method, double disc synergy test (DDST), and ESBLNDP test. All confirmed *ESBL*-producing isolates were screened for the *bla*$_{CTX-M}$ gene.

Results: Overall, 49% of isolates were *ESBL* positive using the DDST while ESBL-NDP test revealed 47.6% *ESBL* producer. Two isolates (1.4%) gave false positive results with NDP test which indicated the presence of over expressed cephalosporinases (Amp C) with or without the presence of *ESBL*. The sensitivity and specificity of ESBL-NDP test were 97.2% and 100%, respectively in comparison with the DDST whereas the positive and negative predictive values of NDP test were 100% and 97.4%, respectively, with accuracy 98.6%. In our study *CTX-M* genotype could be identified in 83.3% of *ESBL* confirmed isolates.

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

bla $_{CTX-M}$ gene was the predominant gene in ESBLs among Egyptian patients.

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 **(Taha et al., 2015).** Organisms carrying *ESBL* enzymes often display co-resistance to other

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antibiotics including aminoglycosides, quinolones, trimethoprim-sulfamethoxazole and tetracycline. This can cause both community and hospital acquired infections, which represents a major challenge to combat **(Manyahi et al., 2017).**

The resistance acquired by *ESBL* producing strains stems from genetic point mutation and are derived mainly from the *bla* $_{TEM}$ or *bla* $_{SHV}$ types of β-lactamases**,** other types include *bla* $_{CTX-M}$, *bla* $_{VEB}$, and *bla* $_{GES}$ enzymes **(Varkey et al., 2014).** Among them, the highest number of variants described in the last years corresponds to the *CTX-M* family**.**This dramatic spread of *CTX-M*s around the world has been referred as the "*CTX-M* pandemic" due to their increasing description worldwide **(Canton et al., 2012)**.

Over 1.5 billion people are colonized with *ESBL*-producing *Enterobacteriaceae* by one estimate **(Woerther et al., 2013)**. The majority of this burden falls on the developing countries, but the prevalence of *ESBL*-producing organisms is also increasing in the developed communities **(Doi et al., 2017).** *bla*$_{CTX-M}$ group *ESBLs* are now by far the most common *ESBLs* globally, both in the developing and developed worlds **(D'Andrea et al., 2013).**

According to "Antimicrobial resistance: global report on surveillance 2014" published by the World Health Organization (http://www.who.int/drugresistance/documents/surveillancereport/en/), rates of *ESBL* producers among *E. coli* clinical isolates in Egypt are estimated to range from 25-50%,. This increase has coincided with a shift in the *ESBL* types from *bla* $_{TEM}$ and *bla* $_{SHV}$ to *bla* $_{CTX-M}$ group, and spread of *ESBL*-producing *E. coli* into the community **(Doi et al., 2017).**

Although Detection of *ESBL*-producing bacteria by phenotypic methods is a vital step for appropriate management of patients but it may take up to 24-48 h **(Gazin et al., 2012)**. Genotypic identification of these enzymes provides essential information for infection prevention and control efforts, aiding to prevent cross-transmission to other patients (**Pitout** and **Laupland, 2008)**. Therefore, the current study aimed for rapid detection of *ESBLs* by ESBL-NDP test, and to examine the prevalence of *bla* $_{CTX-M}$ gene among *Enterobacteracea* members in Egypt.

Material and Methods:-

Bacterial Isolates:-

A total of 147 Gram negative *Enterobacteracea* isolates were obtained from different clinical samples; urine (78; 53.1%), ascetic fluid (29; 19.7%), blood (20; 13.6%), and both sputum and pus were recovered from (10; 6.8%) samples. Samples were collected from patients admitted to Theodor Bilharz Research Institute hospital during the period from March 2016 to August 2016.

Phenotypic Diagnostic tests:-

Antimicrobial Susceptibility Testing:-

Samples were initially cultured on MacConkey agar medium (Oxoid, England). Identification of bacterial isolates to genus and species level was done by API-20E (Biomerieux, France).

All *Enterobacteriaceae* isolates were tested for detection of *ESBL* production by observing reduced zones of inhibition around third generation cephalosporins discs (disc diffusion test). Such strains were considered to be "suspicious for *ESBL* production" according to Clinical and Laboratory Standards Institute (CLSI) guidelines **(CLSI, 2016).** Confirmatory Double disk synergy test (DDST) was performed to confirm *ESBL* production as described by **Jarlier et al. (1988).** *Klebsiella pneumonia* ATCC 700603 was used as a control strain for a positive *ESBL* production and *Escherichia coli* ATCC 25922 was used as a negative control for the *ESBL* production according to the guidelines of CLSI **(CLSI, 2016).**

The ESBL-NDP (Nordmann/ Dortet/ Poirel) test:-

This is a novel phenotypic detection of *ESBL* enzymes by colorimetric method. The test identifies the hydrolysis of the lactam ring of cephalosporin (cefotaxime), which generates a carboxyl group, by acidifying the culture media. The change in pH resulting from this hydrolysis is identified by the color change generated using a pH indicator (phenol red). Inhibition of *ESBL* activity is evidenced by adding tazobactam.

A single full 10 µl calibrated loop of the studied bacterial colonies was suspended in 150 µl of 20 mM Tris-HCl lysis buffer (Thermo Scientific, USA) in three 1.5 ml eppendorf tubes (A, B, and C) . Eppendorfs were vortexed for 30 min at room temperature (Cole-Parmer, USA) for the mechanical lysis of bacteria. Phenol red solution was prepared

using 2 ml of concentrated (pH 8) phenol solution to which 16.6 ml of distilled water was added. Ten microliters of a concentrated tazobactam solution (Sigma-Aldrich, France) (40 mg/ml) was added to tube C. Next, 100 µl of the solution containing pH indicator (phenol red) was added to tube A (Control), and 100 µl of the same solution supplemented with cefotaxime (6 mg/ml) was added to tubes B and C. The three tubes were incubated at 37°C for 20 min. The results were considered negative when all tubes were red and thus interpreted as containing non-ESBL strains. When tube B was yellow/orange and both tubes A and C were red, the test result was considered positive (*ESBL*-producing isolate). When tube A turned to yellow/red, the test result was considered non interpretable, regardless of any color change for tubes B and C **(Nordmann et al.**, **2012)**.

Genotypic identification of *ESBLs:*-

DNA Extraction:-

All phenotypically confirmed *ESBL* positive isolates by DDST were analyzed for $bla_{CTX-M.}$ Extraction of DNA from a single colony of cultured bacteria was done by Thermo Scientific Gene JET Genomic DNA purification kit; K 0721(Thermo Fisher Scientific, USA).

PCR Assay:-

The PCR reaction was performed in a final volume of 50 µL with a 5 µL of DNA template, 0.5 µM of each of the forward and reverse primers, 25 µL of master mix (DreamTaq Green PCR Master Mix, Thermo Scientific, USA) and the volume was completed to 50 µL by using 19 µL of sterile nuclease free water. Primers used were consensus designed to catch all groups of *CTX-M* gene and were described by **Pagani et al., 2003 (Table 1)**. Amplification reactions were carried out in Biometra Germany thermal cycler, under the following conditions: initial denaturation at 95ºC for 3 min, followed by 34 cycle of denaturation at 95ºC for 30 sec, annealing at 49ºC for 40 sec and elongation at 72ºC for 40 sec. The final elongation step was extended to 10 min at 72ºC. The amplified products of the PCR reactions were analyzed by electrophoresis in 2% agarose gel with ethidium bromide, and 100 bp ladder used as DNA molecular weight standard. In each PCR assay, a negative control (lacking DNA) was included.

Table1:-PCR primers used for amplification of *CTX-M* gene.

Amplified gene	Primers (5`-3`)	Amplicon size (bp)	Reference
CTX-M	F: ATGTGCAGYACCAGTAARGT R: TGGGTRAARTARGTSACAGA	593	**Pagani et al., 2003**

Results:-

DDST had classified the tested strains into (72/147; 49%) *ESBL* producers and (75/147; 51%) non-*ESBL* producers. On the other hand, NDP test revealed (70/147; 47.6%) ESBL producers among the examined *Enterobactericeae* isolates. Two isolates (2/147; 1.4%) - one *E. coli* and one *K. pneumonia* strains - gave results which indicated the presence of over expressed cephalosporinases (Amp C) with or without the presence of *ESBL* **(Table 2; Fig. 1)**. They were recorded as false positive results. *CTX-M* genotype could be identified in 83.3% of all *ESBL* confirmed isolates (60/72) **(Table 3; Fig.2)**. *E.coli* showed (48/72; 66.6%) positive result for *ESBL* producing strains **(Table 2)**, among which bla_{CTX-M} type *ESBL* producers were found in 66.7% of *E. coli*, 26.7% of *K. pneumonia* and 6.6% of *K.oxytoca* **(Table 3)**. Both isolates detected by *ESBL*-NDP test that were reported to be Amp-C didn‟t found to carry the $bla_{CTX-M.}$

Table 2:-Frequency of *Enterobacteriaceae* species included in the study and their *ESBL* production rates

Organism (n=147)	**DDST test**		**ESBL NDP test**		
	ESBL (n= 72)	Non-ESBL (n= 75)	ESBL(n= 70)	Non-ESBL (n= 75)	Amp C (n=2)
***E. coli* (n= 85)**	48 (66.6%)	47 (49.3%)	47 (67.2%)	37 (49.3%)	1 (50.0%)
***K. pneumonia* (n= 39)**	20 (27.8%)	19 (25.4%)	19 (27.1%)	19 (25.4%)	1 (50.0%)
***K. oxytoca* (n= 12)**	4 (5.6%)	8 (10.7%)	4 (5.7%)	8 (10.7%)	0 (0.0%)
***Enterobacter* (n= 7)**	0 (0.0%)	7 (9.3%)	0 (0.0%)	7 (9.3%)	0 (0.0%)
***P. mirabilis* (n= 3)**	0 (0.0%)	3 (4.0%)	0 (0.0%)	3 (4.0%)	0 (0.0%)
***Citrobacter* (n= 1)**	0 (0.0%)	1 (1.3%)	0 (0.0%)	1 (1.3%)	0 (0.0%)

Data are expressed as number (%).

The sensitivity and specificity of *ESBL*-NDP test were 97.2% and 100%, respectively in comparison with the DDST whereas the positive and negative predictive values of NDP test were 100% and 97.4%, respectively, with accuracy 98.6%. Kappa testing showed an almost perfect agreement between the *ESBL*- NDP test and DDST in detecting ESBLs ($K^{\#}$ 0.973 = very good agreement).

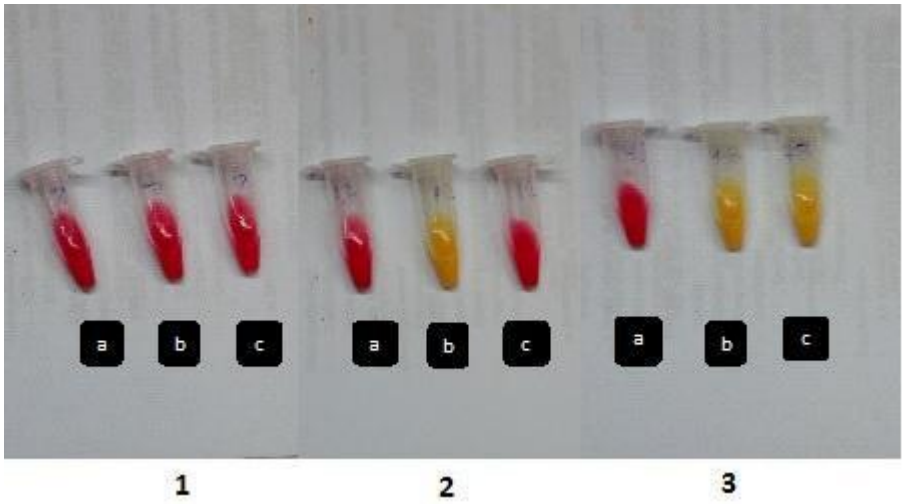

Fig. 1:-NDP test results, 1=negative (non *ESBL*) 2= positive (*ESBL*) 3=Amp-c ± *ESBL*

1. Eppendorf a (bacterial suspension without antibiotic)
2. Eppendorf b (bacterial suspension with cefotaxime)
3. Eppendorf c (bacterial suspension with cefotaxime+ tazobactam).

Table 3:-CTX-M (+/-) in ESBL Enterobacteriaceae species.

Organism	***CTX-M* consensus gene**		**P value**
	Positive (n= 60)	**Negative (n= 12)**	
E. coli (n= 48)	40 (66.7%)	8 (66.7%)	0.619
K. pneumonia (n= 20)	16 (26.7%)	4 (33.3%)	
K. oxytoca (n= 4)	4 (6.6%)	0 (0.0%)	

Data are expressed as number (%).
P > 0.05= not significant.

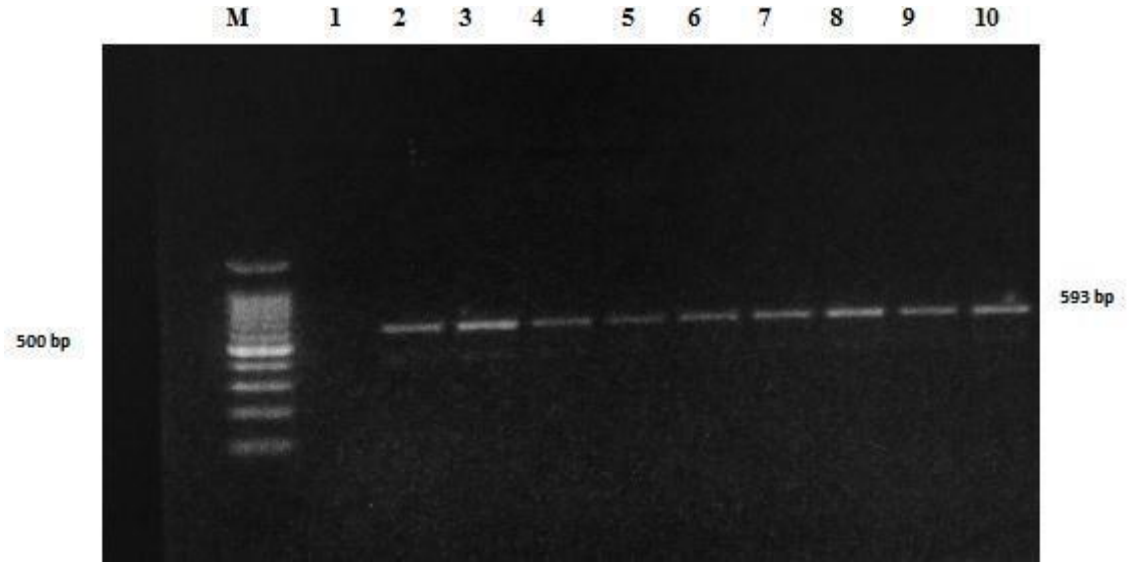

Fig. 2:-Agarose gel (2%) electrophoresis for PCR products of *CTX- M* gene. Lane M: Molecular weight marker (100- 1000 bp). Lane 1: Negative control. Lane 2: Positive control. Lanes 3- 10 positive *CTX-M* gene showing bands at 593 bp.

Statistical analysis:-

Results are expressed as number (%). P value: 0.05 was considered significant and < 0.01 was considered highly significant. Agreement between the different diagnostic tests was tested using kappa statistic. Data analysis was done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 19 windows.

Discussion:-

ESBL production is among the commonest antibiotic resistance problem that has been accelerating worldwide **(Oli et al., 2017)**. The frequent use of β-lactamases has given rise to continuous spread of resistant isolates due to their selective force driving alteration of the resistance mechanisms. Egypt has been recognized as one of the countries with extremely high rate of *ESBL* producers, with up to 70% of isolates producing the enzyme **(Borg et al., 2006)**. Possibly, this high prevalence is related to the less controlled use of antibiotics in Egypt, where many drugs are still available over the counter **(Taha et al., 2015).**Therefore, establishing a new and rapid approach in diagnosis of *ESBL* is crucial in controlling resistance spread. In addition, data concerning dispersion and clonality of *CTX-M* producing isolates, and molecular epidemiology, arouse attention about rapid spread of *CTX-M* resistant genes and the high rate of horizontal transfer between different bacterial species **(Cantón et al., 2012)**.

Therefore, we aimed to detect the burden of *ESBLs* by the rapid *ESBL*-NDP test, and to examine the prevalence of *CTX-M* gene among *Enterobacteracea* in Egypt.

In the present study, *ESBL* production among *Enterobacteriaceae* clinical isolates was found to be 49 % by the DDST, which is nearly identical to 48.9% that was detected by **Abdallah and colleagues (2015)** at El Ahrar Hospital-Zagazig governorate. Comparable results were also obtained by researchers at Assiut University Hospital **(Thabit et al., 2011)** and Benha University Hospital **(Khater and Sherif, 2014)**, as they reported prevalence rates of 52.9% and 53.3%, respectively. Other inter-country studies show a wide and statistically significant degree of variation. Higher results of *ESBL* prevalence of 65.8% were obtained from a previous study by **Ahmed et al. (2009)** in Assuit University Hospitals. This may be due to the fact that all the samples were obtained from ICU patients, which are likely affected by resistant strains rather than patients in other departments. In addition, results of 85.2% and 88.6% were obtained by **El-Badawy et al. (2017)** and by **Fattouh et al. (2017)** in Sohag University respectively. On the other hand, a former study by **Fam et al. (2011)** reported that *ESBL* producing ***Enterobacteriaceae*** isolated from clinical specimens represented 29.9%. Various factors may contribute for this difference as the prevalence of *ESBL*-expressing bacteria varies across different geographical regions, moreover, it depends on antimicrobial stewardship programs, and infection control practices in different hospitals.

Fewer than 10% of isolated strains express *ESBLs* as in USA **(Castanheira et al., 2013),** Canada **(Simner et al., 2011),** Japan, and Korea **(Yan et al., 2014).** The level of resistant bacteria is higher in Africa and is comparable to those detected in Egypt. Ghana revealed resistance rate of (49 %) **(Obeng et al., 2013),** Cameroon (54 %) **(Schaumburg, et al., 2013)**, Gabon (45 %) **(Magoué et al., 2013),** and Morocco (43%) **(Girlich et al., 2014).**

Although *Klebsiella* spp. were more frequently recognized as *ESBL* producers in other studies **(Chander and Shrestha, 2013),** in the current study, 66.6% of *E. coli*, and 33.4% of *Klebsiella species* (*K. pneumoniae* 27.8%, *K. oxytoca* 5.6%) isolates were *ESBL* producers. A study performed by **Kateregga *et al.* (2015),** also reported that *E. coli* was the most common *ESBL* producer (53.9 %) isolated from urine samples, followed by *K. pneumoniae* (28.7 %)**.** Similar predominance of *ESBL* producing *Escherichia coli* has been reported by **Pant et al. (2016)** from Nepal, **Sharma et al. (2013)** and **Shanthi and Sekar (2010)** from India.

In this work, we used another phenotypic method; the *ESBL*-NDP test in addition to the DDST. *ESBL*- NDP test was able to detect 70 out of 72 ESBL producers while the other 2 were Amp C. The sensitivity and positive predictive value of NDP test were 97.2% and 100% respectively in comparison with the DDST. This result was nearly similar to those of **Nordmann et al. (2012)** who evaluated the *ESBL*-NDP test on 255 strains, where the sensitivity of the test was 92.6%. Another study **Dortet et al. (2014)** who applied the *ESBL*-NDP test on 500 ESBL producing *Enterobacteriaceae* recovered from urine samples, reported that the sensitivity of the *ESBL*-NDP test was 98% while the positive predicative value was 98%.

On the other hand, **Taha and colleagues (2015)** in Suez- Canal University reported that 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 predictive values of this test were 95.8 and 100%, respectively. The inconsistency of the results may be due to differences according to region or country, also several risk factors have been documented to be associated with *ESBL* acquisition, including: previous hospitalization, previous use of antibiotics such as third generation cephalosporins, and hospital overcrowding **(Sonda et al., 2016)**. The detection of *ESBLs* in Amp-C producing Gram Negative Bacteria (GNB) is problematic, therefore, bacterial pathogens producing both *ESBLs* and Amp-C β- lactamases create a requirement for laboratory testing methods that can accurately detect the presence of these enzymes in clinical isolates **(Pitout et al., 2003)**. Our work demonstrated the presence of two false positive isolates detected by the *ESBL*-NDP test as some isolates could contain combined *ESBL* and Amp-C producing enzymes giving a positive result. The same result was obtained by another study at Suez- Canal University were *ESBL*-NDP test detected also two Amp-C samples **(Taha et al., 2015).**

In our study, the specificity and the negative predictive value of the *ESBL*-NDP test were 100% and 97.4%, respectively, which were comparable with **Nordmann et al. (2012)** and **Dortet et al. (2014)** as they were 100% and 99.8 respectively. In addition, Kappa testing had an excellent concordance between the *ESBL*-NDP test and DDST in detecting *ESBLs* (K= 0.973).

Many studies reported that *CTX-M* genotypes has been widely increased, and was the most predominant *bla* resistant gene among *Enterobacteriaceae*. Moreover, it has been the most encountered gene in different geographical regions of the world including Middle East Arab countries **(Badran et al., 2016; Baroud et al., 2011).**

In our results, *bla* $_{CTX-M}$ gene was detected in 83.3% of *ESBL* producers by conventional PCR. The frequency of *bla* $_{CTX-M}$ gene in *E. coli* was 66.7%, but in *K. pneumonia* and *K. oxytoca* it was 26.7% and 6.7% respectively. This is consistent with the results obtained in Benha Governorate by **Saeed et al. (2017),** as 71.8% of the *ESBL*-producing isolates were of the *bla* $_{CTX-M}$ type. Also, many studies reported that *CTX-M* was the most prevalent *ESBL* gene type in Egypt **(Abdallah et al., 2015; Fattouh et al., 2017; Abdel-Moaty et al., 2016)**. In contrast to our findings, **Ahmed et al. (2009)** reported that *bla* $_{TEM}$ was the most frequent β-lactamase encoding gene. Worldwide, *bla* $_{CTX-M}$ gene was the most detected resistance gene in Turkey and its prevalence in *E. coli* was reported to be 63.9% **(Peirano and Pitout 2010)**. Furthermore, *bla* $_{CTX-M}$ gene was detected in 80% 0f *E.coli* proved to be *ESBL* in India **(Sharma et al., 2013)**. **Alfaresi** and colleagues **(2011)** highlighted the emergence and dissemination of *CTX-M-15* producing *E. coli* and *K. pneumoniae* in the United Arab Emirates (UAE) demonstrating that the majority of the strains (87%) in their study expressed the *CTX-M* gene which complies with the present study. These findings agree with other contemporary studies from around the world that show that *ESBL* genes of the *CTX-M* type are dominant **(Livermore et al., 2007; Cantón et al., 2012; Burke et al. (2016)**].

Conclusion:-

In conclusion, there is high prevalence of *ESBL* producing strains in our hospital. The *ESBL*-NDP test leads the DDST in its rapid ability for detection of *ESBL* production from bacterial cultures. It is also easy to prepare with cheap in-hand kits, making it a better alternative for *ESBL* detection in small laboratories. A positive result in the *ESBL*-NDP test may indicate exclusion of expanded-spectrum cephalosporins for treating such patients. In addition, our study clearly demonstrates the striking change in gene pool of *Enterobacteriaceae,* as higher rates of *bla* $_{CTX-M}$ detection confirms mobilization of these genes via plasmids replacing *bla* $_{SHV}$ and *bla* $_{TEM}$ resistance genes.

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