

Phenotypic and genotypic characterization of Extended Spectrum β -lactamases producing *Proteus mirabilis* isolates.

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Received on: 28. 06. 2021

Revised on: 01. 07. 2021

Accepted on: 04. 07. 2021

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ABSTRACT

Background: The Extended spectrum β -lactamases (ESBLs) production is one of the most essential mechanism of drug resistance in *Proteus mirabilis* (*P. mirabilis*). **Objective:** Phenotypic and genotypic detection of extended spectrum β -lactamases production in *P. mirabilis* and its antimicrobial resistance among clinical isolates. **Methods:** A total of Sixty *P. mirabilis* clinical isolates were tested for Extended spectrum β lactamase production by modified double disc synergy test (MDDST). Polymerase chain reaction (PCR) was done to detect Extended spectrum β -lactamases genes in the clinical isolates. Antimicrobial susceptibility testing was done by means of Kirby Bauer disc diffusion method. **Results:** Out of the 60 *P. mirabilis* clinical isolates, 17 isolates (28.33%) were ESBL producers by phenotypic test. Genotypically; ESBLs genes were detected and the most prevalent ESBL resistance gene was TEM (91.7%) followed by SHV (75%), CTX-2-59 (56.7%) and CtxM-15 (51.7%). Moderate level of resistance to ofloxacin, ciprofloxacin, amoxicillin/ clavulanic acid, cefotaxime and ceftazidime (60.0%, 58.3%, 45.0%, 41.7% and 40.0% respectively) was recorded. In addition, 73.33% of isolates were classified as multidrug resistant (MDR). **Conclusion:** Monitoring of Extended spectrum β -lactamase producing *P. mirabilis* is very important because of its high prevalence among urinary tract infections. Also; increasing awareness for clinicians and enhancing laboratories tests leading to reduce the spread of these isolates.

Keywords : *P. mirabilis*, Extended spectrum β -lactamase, antimicrobial resistance, urinary tract infections.

INTRODUCTION

Proteus mirabilis (*P. mirabilis*) is a Gram-negative rod-shaped bacterium frequently noted for its swarming motility and urease activity. It considers as an important uropathogen between patients with complicated urinary tract, urolithiasis and extended-term urinary catheterization (CAUTI). These infections might be developed to renal stones due to alkalization of urine. Presence of ammonia resulted from urease-catalyzed urea hydrolysis leading to magnesium and calcium crystallization which could obstruct the lumen of indwelling catheters (Ali and Yousif, 2015, Armbruster *et al.*, 2018).

Cephalosporins and β -lactam/ β -lactamase inhibitors were found to be effective against *P. mirabilis*. Though, strains resistant to β -lactams intermediated by developed β -lactamases appeared in 1990s. Plasmid borne extended-spectrum β -lactamases (ESBLs) were the greatest worrisome among these β -lactamases due to their resistance to almost penicillins and cephalosporins and their ability to spread amongst several species of *Enterobacteriaceae*. Numerous reports had represented that ESBLs β -lactamase- *P. mirabilis* producer isolates could initiate clonal widespread. Thus, wide nosocomial outbreaks and community acquired infections were developed. Failure of treatment and clinical morbidity were also extra predictable to happen in patients infected by ESBLs-producing *P. mirabilis*, which had been estimated to inadequate therapy (Wang *et al.*, 2014). Mostly ESBLs were derivatives of *TEM-1* type, *TEM-2* type and *SHV-1* type of β -lactamase, that were confined one point code gene mutations or more. Recently, Increasing studies of *CTX-M*-type ESBLs were developed by *Proteus mirabilis* as well as *TEM*-type ESBLs (Huang *et al.*, 2014). World Health Organization (WHO) reported that the most serious problem was the routine use of antibiotics leading to the incidence of antimicrobial drug resistant and development of antimicrobial resistant genes. *P. mirabilis* has been concerned in several nosocomial infection outbreaks and community acquired infections in different parts of the world. The most predominant classes of antibiotics prescribed for life threatening cases are the 3rd generation cephalosporins, fluoroquinolones and aminoglycosides. Finally, several studies have reported high resistance of *Enterobacteriaceae* including *P. mirabilis* to these classes of antibiotics (Pawar *et al.*, 2018). MDR (Multidrug resistance) recognized as developed

resistance to at least one antimicrobial category agent, XDR (extremely drug-resistant) recognized as resistance to not less than one or two antimicrobial agents (bacterial isolates susceptible to at least two categories) and PDR (pan drug-resistant bacteria) recognized as resistance to all antimicrobial agents in all antimicrobial categories (Magiorakos *et al.*, 2012). In our study to ensure MDR definition, clinical isolates were tested against different antimicrobial agents with variant antimicrobial categories.

Current study aimed to explore the genotype of ESBLs of *P. mirabilis* clinical isolates isolated from urinary tract infection from Urology and Nephrology center, Mansoura University Hospitals, Dakahlia governorate, Egypt, with recognition of their antibiotic resistance.

MATERIALS AND METHODS

Organisms:

• Bacterial isolates:

During the period from October 2016 to April 2017, 300 clinical specimens were collected from Urology and Nephrology center (UNC), Mansoura University Hospitals, Dakahlia governorate, Egypt. All isolates were checked and identified using automated VITEK-2 system (bioMerieux, Marcy l'Etoile, France). The research proposal was approved from research ethics committee of faculty of pharmacy, Mansoura University.

• Standard strains:

For quality control; *Escherichia coli* (ATCC-25922), *Klebsiella pneumoniae* (ATCC-700603), *Klebsiella pneumoniae* (ATCC BAA-1705) and *Klebsiella pneumoniae* (ATCC BAA-1706) were provided by UNC.

Determination of antimicrobial susceptibility pattern of *P. mirabilis* isolates:

Susceptibility to different antimicrobials was determined by Kirby-Bauer disc diffusion technique according to the clinical laboratory standard institute (CLSI, 2017). Eleven antimicrobial discs of various categories were used including; amikacin 30 μ g (aminoglycosides), amoxicillin/clavulanic acid 20/10 μ g (penicillin/ β -lactamase inhibitor), ciprofloxacin 5 μ g, ofloxacin 5 μ g (second generation fluoroquinolone), ceftazidime 30 μ g and cefotaxime 30 μ g (extended spectrum cephalosporins), imipenem 10 μ g and etrapenem 10 μ g (carbapenem),

pipracillin/tazobactam 100/10 µg (anti pseudomonal penicillin/ β-lactamase inhibitor), Trimethoprim/Sulphamethoxazole 25 µg (folate pathway inhibitor), and nitrofurantoin 300 µg (antiseptic drug); all discs were supplied from Bioanalyze ® products, Turkey. In this study, MDR was evaluated as resistance to at least one agent in three or more antimicrobial categories (Tambekar *et al.*, 2006, Magiorakos *et al.*, 2012).

Phenotypic Extended Spectrum β-lactamases (ESBLs) detection using modified double disc synergy test (MDDST):

Screening of ESBLs-producers was done by determination of minimum inhibitory concentration (MIC) for ceftazidime and cefotaxime. If MIC of ceftazidime and cefotaxime was ≥ 2 µg/ml, the isolate is considered ESBL-producer (CLSI, 2017).

MDDST was applied to confirm the ESBLs production among *P. mirabilis* clinical isolates by using a disc of amoxicillin-clavulanate (20/10 µg) along with ceftazidime (30 µg) and cefotaxime (30 µg). *Escherichia coli* (ATCC-25922) was used as a negative control and *Klebsiella pneumoniae* (ATCC-700603) was used as a positive control for the ESBL production (Kaur *et al.*, 2013).

Detection of Extended Spectrum β-lactamase genes using PCR:

The nucleotide sequence, product size and annealing temperature of the primers used in detection of resistance genes illustrated in table (1). The PCR amplification was performed in a total volume of 25 µl containing 12.5 µl of DreamTaq™ Green PCR master mix, 1 µl DNA extract, 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM) and 9.5 µl of nuclease free water. PCR was performed in Master cycler eppgradient S thermacycler (Epindorf, Mississauga, ON, Canada) as well as a negative control reaction with each primer. The PCR reactions for each gene was performed according to the cycling conditions listed in the following table (2).

STATISTICAL ANALYSIS

To collect descriptive results, data were tabulated, coded, and evaluated using the computer software SPSS version 26.0. The following descriptive statistics were calculated: Interquartile distribution and median (IQR) and Recurrence (Number-percent).

Analytical statistics: The magnitude of variance was checked using one of the following tests in the statistical comparison between the various groups: Mann Whitney, Pearson's chi square test (X^2 -value) or fisher exact, and Monte-Carlo. Spearman's correlation coefficient test was used correlating different parameters. A P value <0.05 was considered statistically significant.

Table (1): The primer sequences of Extended Spectrum β-lactamase gene detection of 60 *P. mirabilis* clinical isolates (Gharrah *et al.*, 2017).

Target gene	Type	Nucleotide sequence (5' to 3')	Product size	Annealing temperature
<i>Ctx M-15</i>	F	GTGATACCACTTCACCTC	255	54° C
	R	AGTAAGTGACCAGAATCAG		
<i>CTX-2-59</i>	F	GATGACTCAGAGCATTCG	739	54°C
	R	GTTGGTGGTGCCATAATC		
<i>SHV</i>	F	ACTATCGCCAGCAGGATC	356	58°C
	R	ATCGTCCACCATCCACTG		
<i>TEM</i>	F	GATCTCAACAGCGGTAAG	786	54°C
	R	CAGTGAGGCACCTATCTC		

Table (2): The primer cycling conditions for PCR reactions of 60 *P. mirabilis* isolates.

Target gene	One cycle		Cycling condition			One cycle
	Initial Denaturation	Denaturation	Annealing	Extension	No. of cycles	Final Extension
Ctx M-15	95°C/ 5min	95°C/30 sec	54° C/ 30sec	72°C/ 1min	35 cycles	72°C/ 5min
CTX-2-59			54°C/ 30sec			
SHV			58°C/ 30sec			
TEM			54°C/ 30sec			

RESULTS

Collection, isolation and identification of clinical isolates:

Out of 300 clinical isolates; 60 isolates of *Proteus mirabilis* were identified using automated VITEK-2 system (bioMerieux, Marcy l'Etoile, France). The most prevalent diagnosis of isolated samples was renal stone cases as it represented 16.70% followed by hypospadias cases (13.30%), then post repair of vesicocutaneous fistula (8.33%). The common of cases represented by males (72%).

Determination of antimicrobial susceptibility pattern of all *P. mirabilis* isolates:

The antimicrobial susceptibility patterns of *P. mirabilis* isolates were evaluated by the disc-diffusion method. The lowest level of resistance was observed with amikacin, imipenem, piperacillin/ tazobactam and etrapenem (8.3%, 11.7%, 11.7% and 13.3% respectively). Moderate level of resistance to ofloxacin, ciprofloxacin, amoxicillin/ clavulanic acid, cefotaxime and ceftazidime (60.0%, 58.3%, 45.0%, 41.7% and 40.0% respectively) was observed. The maximum level of resistance was recorded by nitrofurantoin (98.3%) and trimethoprim/ sulfamethoxazole (81.7%). Moreover, 44 (73.33%) isolates were MDR; distributed with the highest percentage in the median age.

Phenotypic detection of Extended Spectrum β - lactamases (ESBLs) by modified double disc synergy test:

Seventeen isolates (28.33%) were found to have MIC of ceftazidime and cefotaxime ≥ 2 μ g/ ml, so they were considered as ESBL-producers and these results were confirmed by MDDST test. The third generation cephalosporins represented the highest percent for resistance among different isolates. By comparing its resistance statistically, 3rd generation cephalosporins is more prevalent than penicillins group (P-value=0.04) and higher than carbapenems group (P-value \leq 0.001). Moreover, penicillins group represented higher percent for resistance than carbapenems among different isolates (P-value=0.002) as illustrated in figure (1).

PCR detection of resistance genes:

Different Extended spectrum beta-lactamase genes (*CtxM-15*, *CTX-2-59*, *SHV*, *TEM*) were detected by PCR. It was found that the most prevalent resistance gene was *TEM* (91.7%) followed by *SHV* (75%), *CTX-2-59* (56.7%) and *CtxM-15* (51.7%) shown in fig. (2).

The relation between phenotypic ESBL-producer and genotypic ESBL resistance genes represented in fig. (3); Although 17 (28.3%) isolates gave positive ESBLs producer by phenotypic test and 43 (71.7%) negative ESBL-producer isolates, but also had verified results against selected ESBL-resistance genes. It has shown no significance P-value.

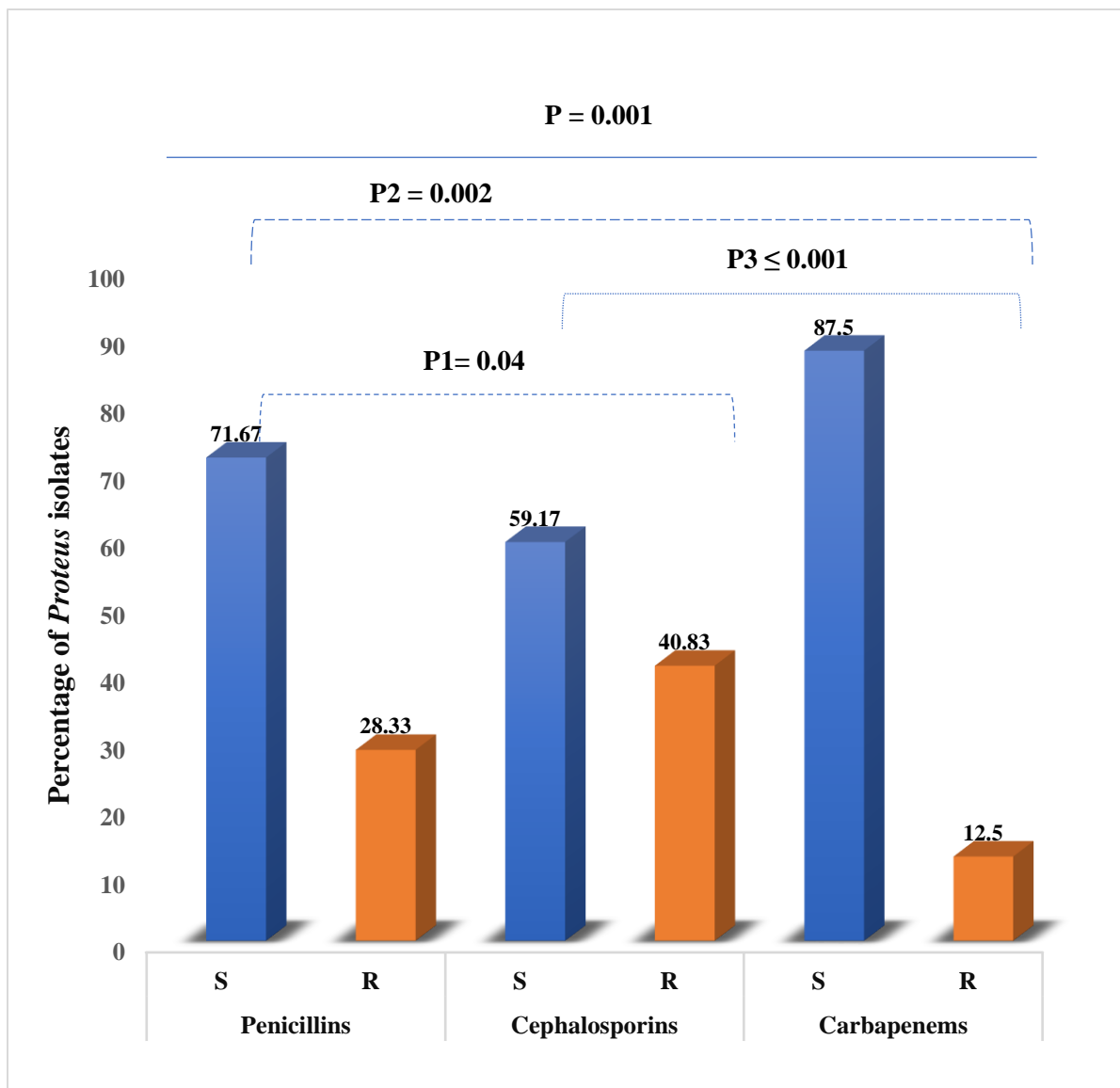
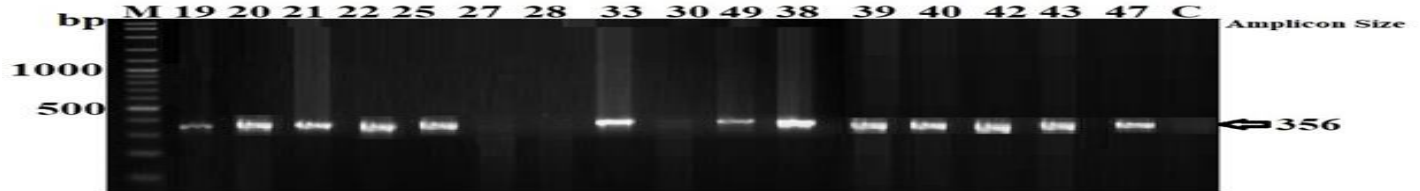


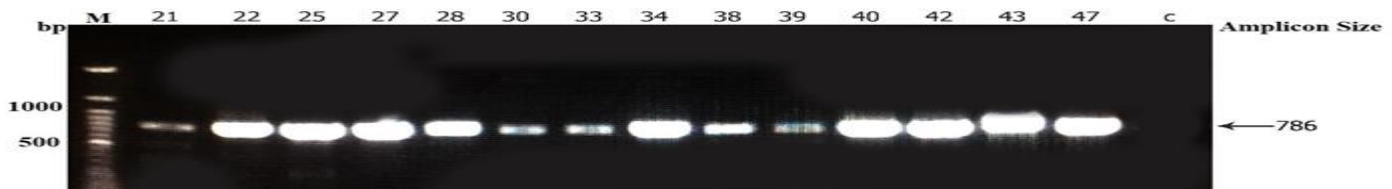
Fig. (1): Resistance percentage of β -lactam classes among different *P. mirabilis* clinical isolates.

- : (P) Probability between all β -lactam classes.
- - - : (P1) Probability between Penicillins & Cephalosporins.
- - - : (P2) Probability between Penicillins & Carbapenems.
- - - : (P3) Probability between Cephalosporins & Carbapenems.

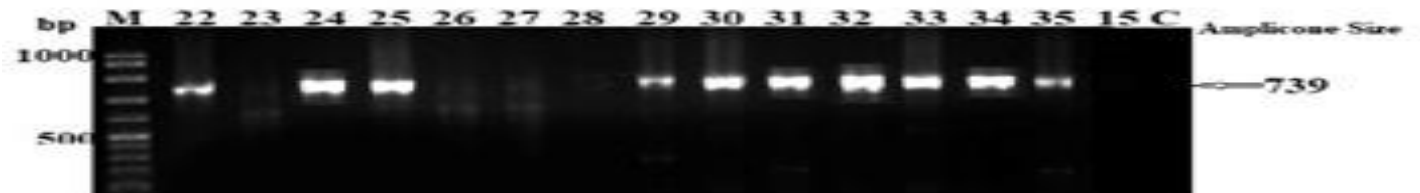
A)



B)



C)



D)



Fig. (2): Agarose gel electrophoresis of different ESBLs genes among *Proteus* isolates. Lane C: negative control (without DNA template), bp: base pair.
A) *SHV*. B) *TEM*. C) *CTX-2-59*. D) *CtxM-15*.

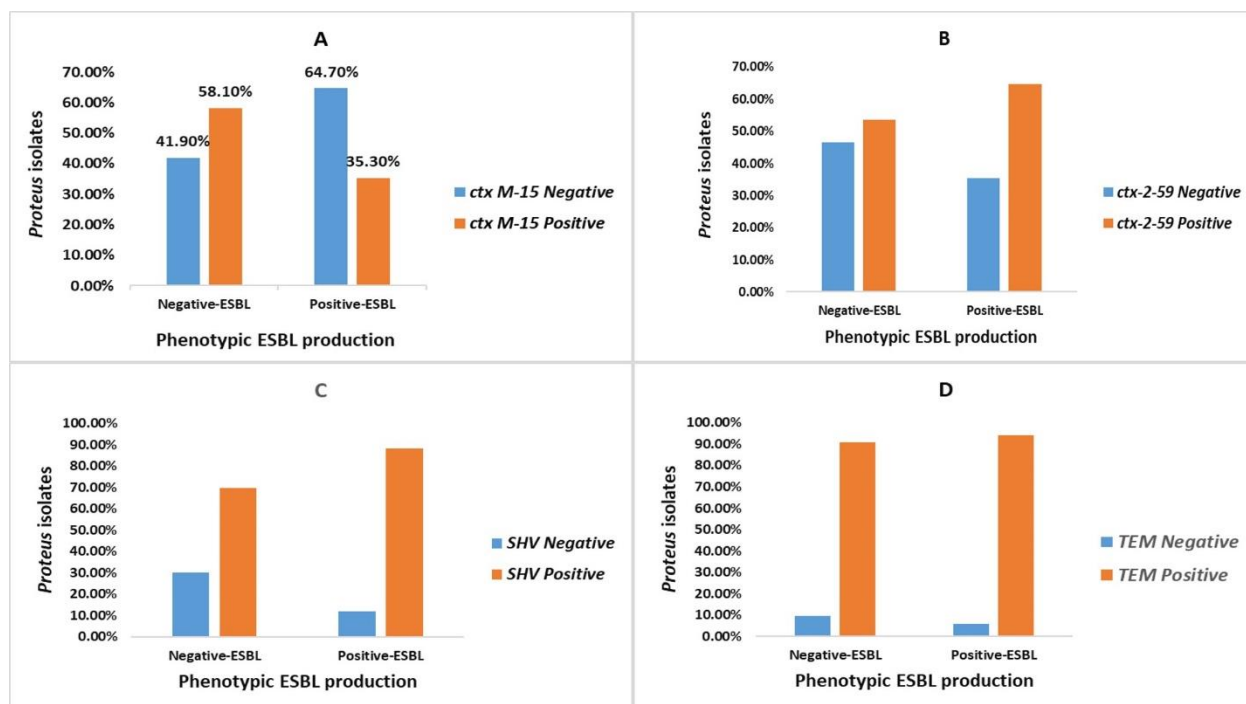


Fig. (3): Distribution of ESBLs genes among positive and negative *Proteus* isolates producers.

A) *CtxM-15*.

B) *CTX-2-59*.

C) *SHV*.

D) *TEM*.

Proteus mirabilis is a Gram-negative bacterium which has been notorious for its ability to swarm firmly across surfaces in a striking bull's eye pattern. Clinically, it exhibits an extraordinary lifestyle as uropathogen invading human urinary tracts. *P. mirabilis* considered as the 2nd greatest predominant uropathogen about 5.2% in the male patients using indwelling catheter (Gravey *et al.*, 2017). It considers as a model microorganism for urease-producing pathogens on indwelling urinary catheters, often leading to poly-microbial infection. Recent studies have illustrated how *P. mirabilis* causes all of these diseases.

In our study; It was found that 44 (73.33%) isolates were MDR, distributed with the highest percentage in the median age. 3rd generation cephalosporins is more prevalent than penicillins group (P-value=0.04) and higher than carbapenems group (P-value \leq 0.001).

Moreover, penicillins group represented higher percent for resistance than carbapenems among different isolates (P- value=0.002).

(Korytny *et al.*, 2016) reported similar results where 55.6% were MDR.

Resemble results of (Danilo de Oliveira *et al.*, 2021); showing that the highest resistance rate was found to trimethoprim/ sulfamethoxazole combination. A study performed by (Gravey *et al.*, 2017) showed different resistance rates for gentamicin (18%), phosphomycin (19%), ciprofloxacin (21%), norfloxacin (22%), nalidixic acid (26%), trimethoprim/ sulfamethoxazole (32%), ampicillin and amoxicillin (40%), and ticarcillin (42%) (Gravey *et al.*, 2017).

In recent years, the infectious diseases treatment has been a significant matter for human security and the continuous increased bacterial resistance elevated patients' expenditures. The ESBLs production has considered as the major hazard due to the usage of the novel generation of cephalosporins.

Enterobacteriaceae rate of production of ESBLs has been significantly elevated (Jain and Mondal, 2008, Ishikawa *et al.*, 2011).

Among *Enterobacteriaceae*, *K. pneumoniae*, *Escherichia coli* and *Proteus mirabilis* were almost vital causative agents of community-acquired infections (Karimisup *et al.*, 2012). Prevalence of infection achieved by ESBLs producing *Enterobacteriaceae* lead to broad use of the cephalosporins generations (Gholipour *et al.*, 2014).

In our study, seventeen (28.3%) *P. mirabilis* clinical isolates gave positive ESBL producer by phenotypic test. In Japan's hospitals, the ESBL prevalence producing strains in *P. mirabilis* varies about 11.9 to 37.8%, depending on the locations and studied reports (Kanayama *et al.*, 2010, Ishikawa *et al.*, 2011). Another study; found that 20% of the prevalence of ESBL-producing *Proteus* has been around, which is similar to prior results (Kurihara *et al.*, 2013).

In our study; The most predominant ESBL resistance gene was *TEM* (91.7%) followed by *SHV* (75%), *CTX-2-59* (56.7%) and *CtxM-15* (51.7%). Another study showed that the most predominant type of ESBLs enzymes were the *bla CTX M* type β -lactamases accounting for about 95% of most types of ESBL-enzymes practically (Gajdacs and Urban, 2019).

From (2000 to 2004), *P. mirabilis* ESBLs producer isolates had been increased gradually in Japan to a highly dramatic level representing 46.2% (Nakano *et al.*, 2012) more elevated than France representing about 6.9% (Chanal *et al.*, 2000) while in United States representing about 9.5% (Saurina *et al.*, 2000). Recently; Nakama study performed from 2013 and 2014 in Japanese hospital and had reported that the prevalence of *P. mirabilis* producing *CTX M2* and *CTX M14* were about 11.1% of the *Proteus* isolates against 11.5% of *E. coli* isolates, and *K. pneumoniae* isolates about 6.2% (Nakama *et al.*, 2016). In Argentina, *CTX M2* ESBLs had represented in *P. mirabilis* (Quinteros *et al.*, 2003), Spain (Mata *et al.*, 2011) and Italy (Pagani *et al.*, 2003). Most β -lactamases donate a dissimilarity resistance to antibiotics involving the 3rd and 4th generation cephalosporins. (Gharrah *et al.*, 2017) confirm that ESBLs producing isolates revealed significantly superior resistance to the tested β -lactamase than non-ESBLs producers ($P < 0.0001$).

Those results were compared with the previous studies by (Shin *et al.*, 2014), wherever ESBLs producing isolates represented advanced significant resistant to almost β lactamase non-ESBLs producing isolates ($P < 0.05$).

Several studies showed that the rate of ESBL-producing organisms was significantly higher among in-patients hospitalized comparing to out-patients ($p < 0.001$). This pattern could be explained by the extensive misuse, overuse, and abuse of different antibiotics by healthcare workers or patients themselves who tend to take antibiotics without medical consultation and prescriptions (Ouchar Mahamat *et al.*, 2019, Sokhn *et al.*, 2020).

Imipenem has considered as the furthestmost effective antimicrobial agent against hospitalized ESBLs producing isolates. The carbapenem antimicrobial agents like imipenem, ertapenem and meropenem, were frequently known as the first drug of choice in the serious infections' treatment generated by ESBL-producing *Enterobacteriaceae* (Mobasherizadeh *et al.*, 2012, Gholipour *et al.*, 2014).

CONCLUSION

It is recommended to monitor ESBL *P. mirabilis* producers. Aminoglycosides and carbapenems has been the drug of choice for infections treatment caused by ESBL producing *P. mirabilis*. Though, they must be prescribed cautiously to avoid loss of outer membrane porin that could change the antibiotic access with marked change of the sensitivity pattern.

Declaration of interest: none

Author Contributions: Lamiaa A. Salama: Methodology, Visualization, Investigation, Writing- Original draft preparation. Hazem Hamed Saleh: Methodology, Investigation. Shaymaa H. Abdel-Rhman: Visualization, Investigation, Writing- Reviewing and Editing. Rasha Barwa: Conceptualization, Visualization, Writing- Reviewing and Editing. Ramadan Hassan: Supervision, Writing- Reviewing and Editing.

This article had not been issued anywhere or currently under consideration by another journal or a publisher.

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