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Detection of *Pseudomonas aeruginosa* harboring bla_{CTX-M} , bla_{OXA} and bla_{SHV} causing infections in Najaf city hospitals

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Abstract

Nosocomial infections caused by *P. aeruginosa* presenting resistance to β-lactam drugs are one of the most challenging targets for antimicrobial therapy. *P. aeruginosa* harboring acquired mechanisms of resistance, such as production of extended-spectrum β-lactamases (ESBLs) have the highest clinical impact. Hence, this report was designed to investigate the presence of genes codifying for ESBLs among *P. aeruginosa* isolated from three hospitals in Najaf city. Thirty-six *P. aeruginosa* isolates were evaluated for the presence of ESBL genes. The isolates presenting ESBL genes were submitted to PCR for molecular evaluation. In all isolates, primary phenotypic test revealed that ESBL phenotype was recognized in isolates (100%) which were expressed resistance to any of the expanded-spectrum cephalosporins and monobactam. Genes encoding ESBLs were detected in 30(83.3%) of isolates. The bla_{CTX-M} was the most prevalent ESBL gene 20(55.6%), followed by bla_{OXA} 18(50.0%) and bla_{SHV} 15(41.7%). These findings raise the concern about the future of antimicrobial therapy, since simultaneous production of ESBLs is known to promote further complexity in phenotypic detection.

Introduction

P. aeruginosa is an opportunistic pathogen responsible for a large spectrum of invasive diseases in healthcare settings, including pneumonia, urinary tract infections and bacteremia (1). In addition, it is a major cause of chronic lung infections and death in children and adults with cystic fibrosis (2). The occurrence of multidrug-resistant *P. aeruginosa* strains is increasing worldwide and limiting our therapeutic options (3). The spread of this organism is often difficult to control, as *P. aeruginosa* exhibit intrinsic resistance to several antimicrobial agents (4).

The production of extended-spectrum β-lactamases (ESBLs) confers resistance at various levels to expandedspectrum cephalosporins, such as cefotaxime and ceftazidime, and to aztreonam, but not normally to the cephamycins and carbapenems (5). These enzymes are encoded by different genes located on either chromosomes or plasmids (6). More recently, appearance of ESBLs such as TEM, SHV, CTX-M, BEL, PER, VEB, GES, PME and OXA-type β-lactamases has become an emergent public health problem, since these enzymes confer resistance to at least all expanded spectrum cephalosporins and compromise efficiency of ceftazidime, an important antibiotic regimen for *P. aeruginosa* (7-8).

The aims of this study were to evaluate the antibiotic susceptibility patterns of *P. aeruginosa* isolated from clinical and hospital environmental specimens at different Najaf hospitals and to identify the genes encoding existence of ESBL genes.

Bacterial isolates and detection of ESBL

Thirty-six *P. aeruginosa* isolates were obtained from hospital environmental samples and patients visited/or admitted to different hospitals in the Holly Najaf province included: Al-Sader Medical City, Al-Hakeem General Hospital, Al-Zahra'a Hospital for Childbirth and Children, over a period between February to October 2011. Isolates were obtained from specimens originated from instrument, floor, burn wound, ear and urinary tract. The isolates were identified by conventional biochemical methods and confirmed by API 20E system.

Antimicrobial susceptibility testing was performed according to the standard method established by the CLSI (9). The isolates were tested in the Kirby Bauer method of disk diffusion to check their susceptibilities to norfloxacin (NX: 5 µg), ciprofloxacin (CIP: 5 µg), amikacin (AN: 30 µg), gentamicin (CN: 10 µg), tobramycin (TOB: 10 µg) aztreonam (ATM: 30 µg), meropenem (MEM: 10 µg), imipenem (IMP: 10 µg), cefepime (FEP: 30 µg), ceftriaxone (CRO: 30 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), ticarcillin- clavulanat (CTC: 75/10 µg), piperacillin-tazobactam (PTZ: 110 µg), ticarcillin (TIC: 75 µg), piperacillin (PRL: 100 µg), carbenicillin (PY: 100 µg) (Himedia-India). Multidrug resistance (MDR) was defined as resistance to 3 or more classes of drugs that would otherwise serve as treatments for *P. aeruginosa* infection (10).

ESBL production was detected by double disk synergy test (DDST) as described by Jarlier(11). Antibiotic disks of cefotaxime (30µg), ceftazidime (30µg), ceftriaxone (30 µg), and azetreonam (30µg) were placed 15 mm (edge to edge) around a central disk of ticarcillin-clavulanate (75/10 µg) on Muller-Hinton agar plates seeded with organism being tested for ESBL production. Plates were incubated aerobically at 37°C for 24 hr. Any augmentation (increase in diameter of inhibition zone) between the central ticarcillin-clavulanate disk and any of the β-lactam antibiotic disks showing resistance or intermediate susceptibility was recorded, and the organism was thus considered as an ESBL producer.

Detection of ESBL types by PCR

P. aeruginosa DNA extraction was done according to the Pospiech and Neumann method (12). Specific primers and protocols were used to detect bla_{TEM}, bla_{SHV} bla_{CTX-M} and bla_{OXA} (13,14,15).

Results

Pseudomonas aeruginosa was isolated from 29 patients and 7 specimens from the hospital environment samples. The frequency of the isolates and their sites of isolation are listed in table (1). Majority of

the isolates were obtained from Al-Sader Medical City (80.6%) followed by Al-Hakeem General Hospital (11.1%), since only a minority of these isolates were from Al-Zahra'a Hospital for Childbirth and Children (8.3%). The frequency of ESBLs producing isolates by disk diffusion assay are summarized in table (1). A ceftazidime resistance was detected in 33 (91.7%) isolates and cefotaxime, ceftriaxone and azetreonam effect were (100%) observed in all isolates. In this study, primary phenotypic test revealed that ESBL phenotype was recognized in all isolates (100%) which were expressed resistance to any of the expanded-spectrum cephalosporins and monobactam. Based on this phenotypic detection, the study revealed a high rate of ESBLs-producing *P. aeruginosa* recovered from clinical and hospitals environmental samples in Najaf.

ESBL production was also screened for using the DDST as a standard disk-diffusion assay. Present results revealed that the isolates did not show any ESBL production as the increased zone size towards the ticarcillin-clavulanic acid disk (Table 2), probably due to the simultaneous production of other type of β-lactamase that could mask the presence of the ESBL (AmpC β-lactamases or inhibitor resistant cephalosporinases). It is noteworthy that the difference in number of the above methods was large. However, the study indicated that no correlation was found between the results obtained with disk diffusion method and DDST.

The molecular method was used to detect the most common kinds of ESBLs; TEM, SHV, CTX-M, and OXA. For this purpose, four types of primers were used with PCR and electrophoresis systems. The distribution of the ESBL genes among isolates is shown in Table (3). One or three *bla*-genes for ESBL were present in 30 (83.3%) isolates. Present results revealed that gene $bla_{\text{CTX-M}}$ was the most incident (alone or in combination), being recovered from 20 (55.6%) of the *P. aeruginosa* isolates (Figure 1), followed by the bla_{OXA} gene (18, 50.0%) and the bla_{SHV} gene (15, 41.7%) as shown in Figures (2) and (3). The most common gene combination among the isolates, were the bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{OXA} gene. Seven (19.4%) isolates had all these three genes present, while 4 (11.1%), 3 (8.3%) and 2 (5.6%) isolates had the combination of a $bla_{\text{CTX-M}}$ +bla_{OXA} genes, bla_{SHV} + $bla_{\text{CTX-M}}$ genes, and bla_{SHV} + bla_{OXA} genes, respectively. Noteworthy, none of the 36 isolates had bla_{TEM} ESBL gene.

Discussion

This report was designed to investigate the presence of genes codifying for ESBLs among *P. aeruginosa* isolated from three hospitals in Najaf city. In recent years, the problem of gradually increasing resistance to antibiotics has threatened the entire world. Production of β-lactamase, which hydrolyses and inactivates β-lactam antibiotics, has been one of the most important resistance mechanisms of many bacterial species, mainly in *P.aeruginosa* and other Gram-negative bacilli (16).The most frequently

encountered ESBLs belong to the CTX-M, SHV, OXA and TEM families. These enzymes producers are usually multiply drug resistant (17). They are mostly plasmid-mediated enzymes capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including third-generation cephalosporins, penicillins and aztreonam (18).

The detection of bla_{TEM} bla_{CTXM}, bla_{OXA} and bla_{SHV} and the unique coexistence of them exemplify the extraordinary ability presented by *P. aeruginosa* to acquire multiple resistance mechanisms. The results showed a high frequency of ESBLs in *P. aeruginosa* isolates 30/36 (83.3%). bla_{CTXM} represents the majority in *P. aeruginosa* isolates 20/36(55.6%). Recent studies indicate that the CTX-M enzymes predominate among the ESBLs of community strains (19, 20, 21). The first CTX-M enzyme described in *P. aeruginosa* and *S. maltophilia* was observed in isolates recovered in 2004 from patient in Amsterdam (22), this enzyme was characterized as CTX-M-1. In the same year, was also observed in *P.aeruginosa* and *Acinetobacter* spp. isolates recovered from Bolivian hospitals (23).CTX-M was also found in a *P.aeruginosa* isolate recovered from hospitalized patient in Brazil in 2005 (24) and in Iran (25,26). Despite of these results, prevalence of CTX-M enzymes in non-fermenter bacill is still rare. This fact could be due to incompatibility of plasmids carrying these genes or might reflect phenotypic difficulties in recognizing non-fermenter isolates expressing CTX-Ms(26). Current report demonstrates that bla_{OXA} (50.0%) is the second most spread among *P.aeruginosa* isolates. The evolution of OXA type ESBLs from parent enzymes with narrower spectra has many parallels with the evolution of SHV and TEM‑type ESBLs (27). *P. aeruginosa* clinical isolates frequently produce narrow and extended-spectrum oxacillinases, whereas *Acinetobacter* spp. does not. In fact, the OXA‑type ESBLs were originally discovered in *P. aeruginosa* isolates (17). Their codification in plasmids and/or transposons may facilitate their horizontal spreading, although many of them have been proved only to have a chromosomal location (27, 28).

In this study, bla_{SHV} gene was detected in 15 of isolates. SHV-1 is a β-lactamase with hydrolytic activity over third and fourth-generation cephalosporins, in addition to penicillins and narrow-spectrum cephalosporins (29). The first report of the SHV-1 in epidemic *P.aeruginosa* strain showed by Kalai *et al*.(30). Seven *P. aeruginosa* isolates producing the clavulanic acid inhibited ESBL SHV-5 were isolated in the hospital in Greece (31). Tavajjohi *et al*.(26) in Iran found four isolates harboring bla_{SHV-1} and bla_{SHV-5}. There are more than 140 SHV varieties described worldwide (32). bla_{TEM} was negative,which could be either due to the absence of specific gene or the presence of other type of genes that could not be targeted by the primers used in this study. Antimicrobial therapy has played an important role in the treatment of human bacterial infections, but the drug

resistance that has emerged in the treatment of bacterial infections due to ESBL enzymes degrades all β-lactam antibiotics and thus bacteria become multidrug resistant (33,).

In conclusion, the ESBL-producing organisms are a breed of multidrug- resistant pathogens that are increasing rapidly and becoming a major problem in hospitals. It is essential to report ESBL production along with the routine sensitivity reporting, which will help the clinicians in prescribing proper antibiotics.

Table (1): Sample demographics and antibiotics susceptibilities of *P. aeruginosa* isolates

Isolate symbol	Hospital	Sample source	Piperacillin	Ticarcillin	Carbenicillin	Ticarcillin - clavulanic	Piperacillin - tazobactam	Ceftriaxone	Ceftazidime	Cefotaxime	Cefepime	Aztreonam	Cefoxitin	Imipenem	Meropenem	Gentamicin	Amikacin	Tobramycin	Ciprofloxacin	Norfloxacin	No. of antibiotic resistant
Pa1	Sh *	BW **	R	R	R	R	R	R	S	R	R	R	R	S	R	R	S	R	S	R	14
Pa2	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	S	R	15
Pa3	Sh	BW	R	R	R	R	R	R	S	R	R	R	R	S	R	R	S	S	R	R	14
Pa4	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R	16
Pa5	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa6	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa7	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa8	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa9	Zh *	IN **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa10	Sh	FL **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa11	Zh	FL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa12	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa13	Sh	Ea **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa14	Sh	IN	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa15	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa16	Sh	FL	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa17	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa18	Sh	Ea	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa19	H*	UR **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa20	Sh	UR	S	R	R	R	S	R	S	R	R	R	R	S	R	R	S	R	S	R	12
Pa21	Hh	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	16
Pa22	Sh	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa23	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa24	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa25	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa26	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa27	Hh	Ea	S	R	S	R	S	R	R	R	R	R	R	S	R	R	S	R	R	R	13
Pa28	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	16
Pa29	Sh	Ea	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa30	Hh	UR	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa31	Sh	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa32	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa33	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa34	Sh	FL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa35	Zh	FL	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa36	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Total resistant (%)			34 (94.4)	36 (100)	35 (97.2)	36 (100)	34 (94.4)	36 (100)	33 (91.6)	36 (100)	36 (100)	36 (100)	36 (100)	12 (33.3)	36 (100)	36 (100)	26 (72.2)	34 (94.4)	31 (86.1)	36 (100)	

***Sh, Al-Sader Medical City; Hh, Al-Hakeem General Hospital; Al-Zahra'a Hospital for Childbirth and Children**
****BW, Burin wound; Ur, Urine; Ea, Ear; IN, Instrument; FL, Floor.**

Table (2): Comparative between initial screening and double disk synergy test for ESBL production by *P. aeruginosa* isolates

No. (%) of Resistance by disk diffusion test				Double disk synergy test positive NO. (%)
Ceftriaxone	Ceftazidime	Cefotaxime	Aztreonam	
36 (100)	33 (91.7)	36 (100)	36(100)	0 (0.0%)

Table (3): Pattern of distribution of ESBL genes among *P. aeruginosa* isolates

Type of *bla*-gene	No. (%) of isolates	Isolate code No.
$bla_{SHV} + bla_{CTX-M} + bla_{OXA}$	7 (19.4)	Pa4, Pa7, Pa14, Pa18, Pa20, Pa24, Pa27
bla_{CTX-M}	6 (16.7)	Pa8, Pa13, Pa19, Pa31, Pa23, Pa33
bla_{OXA}	5 (13.9)	Pa6, Pa15, Pa17, Pa34, Pa35
$bla_{CTX-M} + bla_{OXA}$	4 (11.1)	Pa22, Pa29, Pa30, Pa36
$bla_{SHV} + bla_{CTX-M}$	3 (8.3)	Pa3, Pa10, Pa28
bla_{SHV}	3 (8.3)	Pa9, Pa16, Pa25
$bla_{SHV} + bla_{OXA}$	2 (5.6)	Pa11, Pa32
ESBL genes negative isolates	6 (16.7)	Pa1, Pa2, Pa5, Pa12, Pa21, Pa26

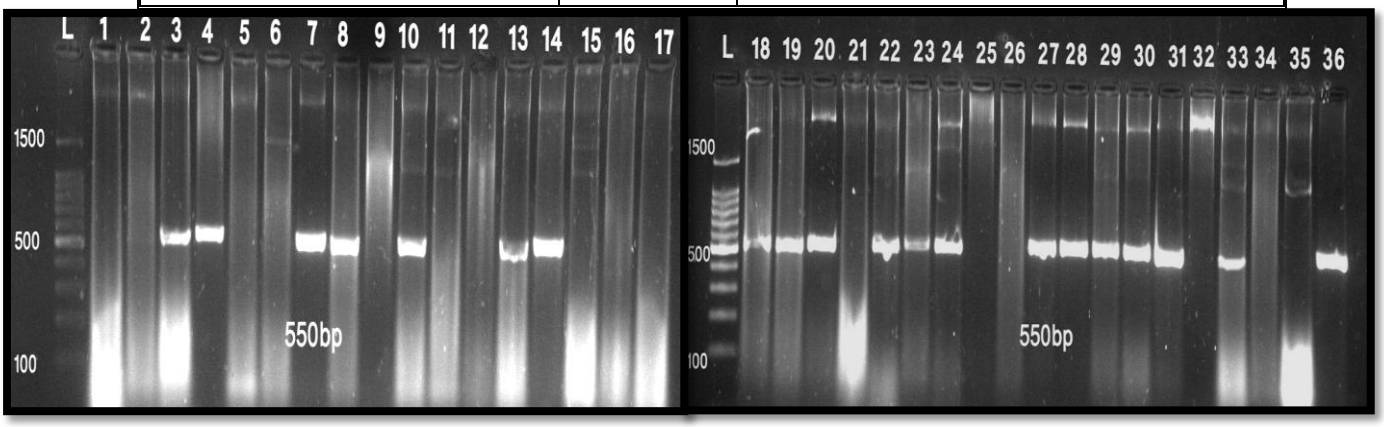

Figure (1 a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of carbapenem resistant *P. aeruginosa* isolates and amplified with primers bla_{CTX-M} forward and bla_{CTX-M} reverse. show positive results with bla_{CTX-M} gene (550 bp).

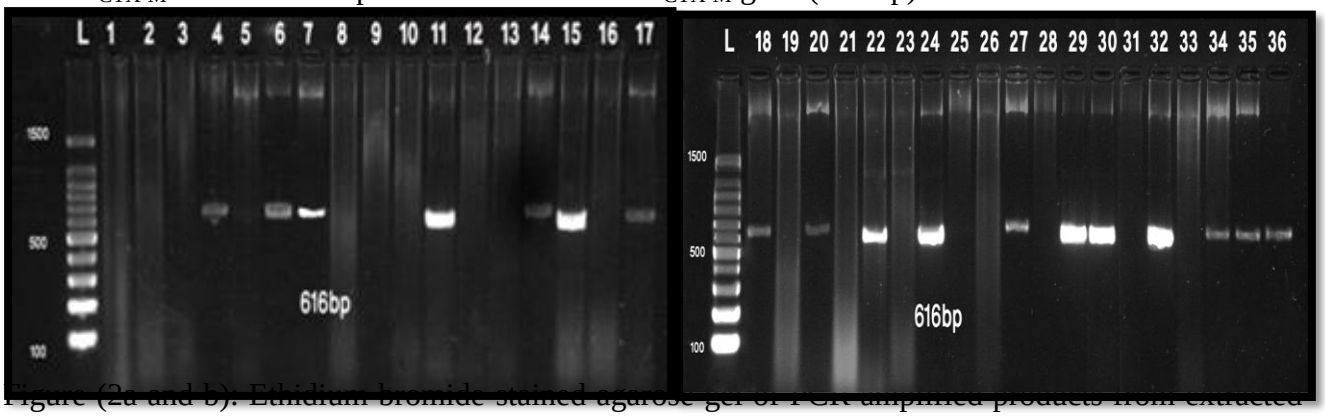

Figure (2a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of carbapenem resistant *P. aeruginosa* isolates and amplified with primers bla_{OXA} forward and bla_{OXA} reverse , show positive results with bla_{OXA} gene (616 bp).

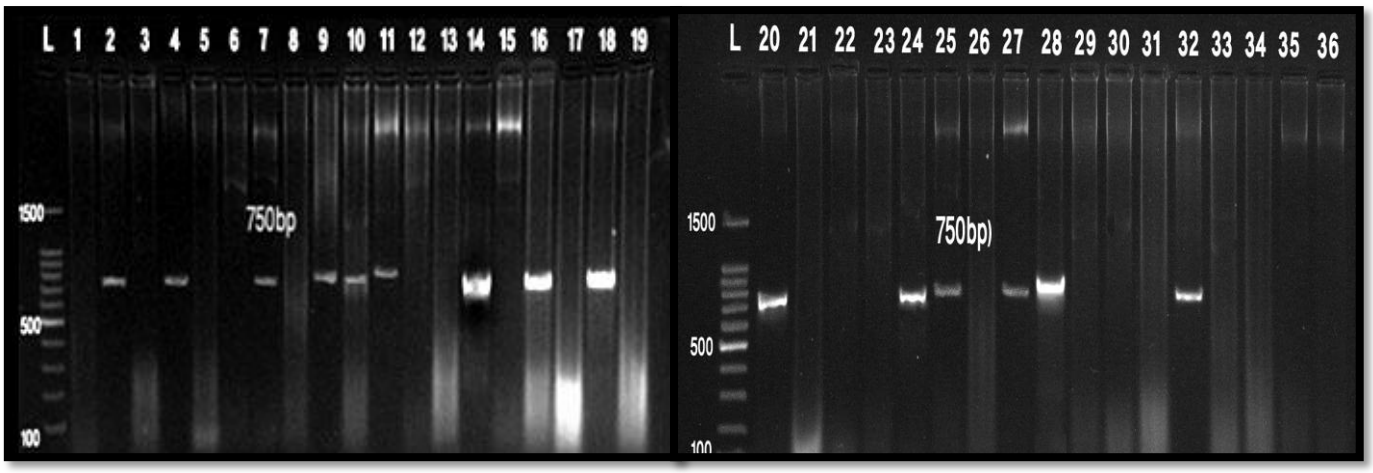

Figure (3a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of carbapenem resistant *P. aeruginosa* isolates and amplified with primers bla_{SHV} forward and bla_{SHV} reverse, show positive results with bla_{SHV} gene (750 bp).

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