

Original Research Article

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PCR for the Detection of Extended Spectrum β -Lactamases Genes of *Pseudomonas aeruginosa*

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ABSTRACT

Extended spectrum β -lactamases are often plasmid mediated and derived from mutations in *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{AmpC} genes by one or more amino acid substitution around the active site. These genes are responsible for resistance to beta lactams in *Pseudomonas aeruginosa*. In this study, *Pseudomonas aeruginosa* were isolated from wound and burn infection patients admitted to Diwanyia Teaching Hospital. Routine microbiological methods were used then PCR technique for the detection of four extended spectrum β -lactamases genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{AmpC}). The PCR results indicated high prevalence of Extended-spectrum β -lactamases in burn infections (*bla*_{TEM} (66.7%), *bla*_{SHV} (86.7%), *bla*_{CTX-M} (93.3%), and *bla*_{AmpC} (53.3%) and less prevalent in wound infections infected with *Pseudomonas aeruginosa* (*bla*_{TEM} (59.1%), *bla*_{SHV} (68.2%), *bla*_{CTX-M} (81.8%), and *bla*_{AmpC} (50%). In conclusion, *Pseudomonas aeruginosa* isolates of wound and burn infection patients are highly associated with emergence of extended spectrum β -lactamases genes that provide antibacterial resistance to almost broad spectrum antibiotics.

Keywords

ESBLs, *Pseudomonas aeruginosa*, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{AmpC}, genes, PCR, MDRPA, Nosocomial infections

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Introduction

Pseudomonas aeruginosa is one of the widely occurred as an opportunistic pathogen, causing outbreaks of nosocomial infections including pneumonia, urinary tract infections, and bacteremia (Bahmani and Ramazanzadeh, 2013; Wirth *et al.*, 2009). Nosocomial infections caused by *P. aeruginosa* are frequently life threatening and often difficult to treat; due to its capacity to develop resistance to multiple classes of antimicrobials through both intrinsic mechanisms and acquisition of transferable resistance determinants (e.g., genes encoding β -

lactamases) (Umadevi *et al.*, 2011; Tian *et al.*, 2011). Eventually leading to the emergence of multidrug-resistant *P. aeruginosa* (MDRPA) strain (Peymani *et al.*, 2017). *P. aeruginosa* (MDRPA) is involved in the production of various classes of extended spectrum β -lactamases (ESBLs) (Bokaeian *et al.*, 2015).

Extended-spectrum β -lactamases (ESBLs) are enzymes that hydrolyze penicillin, monobactam aztreonam (ATM) and extended-spectrum cephalosporins (cefotaxime (CTX), ceftazidime (CAZ) and ceftriaxone) (Jiang *et al.*, 2006). The clavulanic acid is β -lactamase inhibitor

capable of inhibit ESBLs (Rafiee *et al.*, 2014). The extended-spectrum β -lactamases are plasmid-encoded enzymes and the major genetic groups of ESBLs are TEM, SHV, and CTX-M that mediate resistance to wide range of antibiotics' generation (Peymani *et al.*, 2017). On the other hand, *P. aeruginosa* produce chromosomal AmpC-lactamase, which mediate high-level penicillin and cephalosporin resistance (Tian *et al.*, 2011).

Unfortunately, the emergence of extended-spectrum β -lactamases (ESBLs) *P. aeruginosa*, among hospitalized patients in burn units is life-threatening because the burn patient's infection is one of the most complicated issue since these strains are multidrug-resistant and challenging to treat (Radan *et al.*, 2016). So it is obvious that using PCR in the detection of TEM, SHV, CTX and AmpC genes in ESBL-producing bacteria and their pattern of antimicrobial resistance is quite important; For the establishment of the appropriate antimicrobial therapy and for assessment and control of the spread of drug resistant *P. aeruginosa* (Bokaeian *et al.*, 2015; Polotto *et al.*, 2012).

Materials and Methods

Sample collection

50 swab samples were collected from wound and burn, infection from patients of Diwaniyaprovince/Iraq. The samples were placed in sterile transport media then transferred to the Microbiology Laboratory / College of Veterinary Medicine and store in a refrigerator until microbiological processing.

Bacterial isolation

Pseudomonas aeruginosa was isolated from wound and burn samples by inoculation on Brain Heart Infusion Broth medium at 37°C overnight for primary enrichment culture and

then the bacterial growth were inoculated on chrome agar at 37°C overnight for selective isolation of pure culture of *Pseudomonas aeruginosa* isolates.

Bacterial genomic DNA extraction

Bacterial genomic DNA was extracted from *P.aeruginosa* isolates using (Presto™ Mini gDNA Bacteria Kit. Geneaid. USA). One ml of overnight bacterial growth on BHI broth was placed in 1.5ml microcentrifuge tubes and centrifuged at 10000 rpm for 1 minute. The supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was made according to the manufactural instructions. The extracted gDNA was checked by "Nano drop" spectrophotometer, then stored at -20°C in refrigerator until PCR assay.

Polymerase chain reaction (PCR)

PCR was performed for the detection of Extended-spectrum β -lactamases (ESBLs) (*bla*_{TEM}, *bla*_{-SHV}, *bla*_{-CTX-M}, and *bla*_{AmpC} genes) by using specific ESBLs primers designed by using NCBI-GenBank and primer3 plus design online. As shown in table 1.

These primers were provided by (Bioneer Company. Korea). Then PCR master mix was prepared using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250 μ M, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl₂ 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20 μ l total volume by added 5 μ l of purified genomic DNA and 1.5 μ l of 10pmole of forward primer and 1.5 μ l of 10pmole of reverse primer, then completing the PCR premix tube by deionizer PCR water into 20 μ l and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was

performed in a thermocycler (MyGene Bioneer, Korea) by setting up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 60 °C for 30 s, and extension 72 °C for 1 min and then final extension at 72 °C for 10 min. The PCR products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator.

Results and Discussion

A total of 50 swab samples were collected from wound and burn infections; where wound infections were 32 and 18 were burn infections. *Pseudomonas aeruginosa* isolated from 22 (68.7%) of wound infections samples, while in burn infections *Pseudomonas aeruginosa* isolated from 15 (83.3%) of samples, the results are shown in the table 2 (Fig. 1–4).

Molecular detection of ESBLs genes

PCR appear to be specific for the detection of Extended-spectrum β -lactamases (ESBLs) (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{AmpC} genes) producing *Pseudomonas aeruginosa*. The PCR results are shown in the table 3. The results of *bla*_{TEM} genotyping revealed that Thirteen (59.1%) out of 22 isolates and 10 (66.7%) out of 15 isolates were harboring *bla*_{TEM} in wound and burn infections, respectively. The *bla*_{SHV} gene was found in 15 (68.2%) and 13 (86.7%) in wound and burn infections, respectively.

The *bla*_{CTX-M} gene was found in 18 (81.8%) out of 22 isolates of *P. aeruginosa* in wound, while 14 (93.3%) out of 15 isolates in burn infections, finally the results of *bla*_{AmpC} genotyping revealed that 11 (50%) out of 22 isolates and 8 (53.3%) out of 15 isolates out of 15 isolates were harboring *bla*_{AmpC} in wound and burn infections, respectively.

Hospitalized patients burn show increasingly emerging strains of ESBL-producing *P. aeruginosa*, especially; burn patients with prolonged intensive care (Uemura *et al.*, 2010). In this study, the frequency of the ESBL genes including plasmid-encoded (*bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M}) and chromosomal *bla*_{AmpC} among *P. aeruginosa* burn and wound isolates were studied. *bla*_{CTX-M} is the most dominant gene in both burn and wound isolates making 93.3% and 81.8% respectively.

This is in agreement with the results of Celenza who reported a high prevalence of *bla*_{CTX-M} genes (Celenza *et al.*, 2006). *bla*_{CTX-M} product, the plasmid-mediated cefotaximases, most of CTX-Ms unveil influential resistance for cefotaxime and ceftriaxone but not ceftazidime (Zhao and Hu, 2013).

However, the frequency of the *bla*_{CTX-M} group of ESBL in this study is higher as compared with other studies; in a study from Iran *bla*_{CTX-M} gene was detected only in one isolate (Shacheraghi *et al.*, 2010). Another study reported a high prevalence of CTX-M genes in Enterobacteriaceae instead of *Pseudomonas*, as many genetic elements, lead to transfer of *bla*_{CTX-M} genes from the chromosomes to plasmids (Zhao and Hu, 2013). As well as the presence of *bla*_{CTX-M} gene in *Pseudomonas aeruginosa* is more likely result of horizontal transmission from Enterobacteriaceae (Polotto *et al.*, 2012). This may interpret presence the variations within different geographical areas.

TEM, SHV and CTX genotypes are predominant within members of the family Enterobacteriaceae in different Asian countries. The presence these genes in *P. aeruginosa* were described recently (Chen *et al.*, 2015). The present results indicate that 86.7% of isolates that possess *bla*_{SHV} were isolated from burns whereas 68.2% of the isolates were from wounds.

Table.1 The name, sequence and products size of primers

Primer	Sequence		Size of PCR product
<i>bla</i> _{-CTX-M}	F	AGC GAT AAC GTG GCG ATG AA	247bp
	R	TCA TCC ATG TCA CCA GCT GC	
<i>bla</i> _{-SHV}	F	CCG CCA TTA CCA TGA GCG AT	410bp
	R	AAT CAC CAC AAT GCG CTC TG	
<i>bla</i> _{-TEM}	F	GGT GCA CGA GTG GGT TAC AT	531bp
	R	TGC AAC TTT ATC CGC CTC CA	
<i>bla</i> _{-AmpC}	F	AAA CGA CGC TCT GCA CCT TA	670bp
	R	TGT ACT GCC TTA CCT TCG CG	

Table.2 Isolation frequency

Sample type	Total samples	Positive isolate	Percent
Wound	32	22	68.7%
Burn	18	15	83.3%

Table.3 Percent of genes presence among isolates

Virulence gene	Clinical isolates	
	Wound (22)	Burn (15)
<i>bla</i> _{-TEM}	13/22 (59.1%)	10/15 (66.7%)
<i>bla</i> _{-SHV}	15/22 (68.2%)	13/15 (86.7%)
<i>bla</i> _{-CTX-M}	18/22 (81.8%)	14/15 (93.3%)
<i>bla</i> _{-AmpC}	11/22 (50.0%)	8/15 (53.3%)

Fig.1 Agarose gel electrophoresis of PCR products of *bla*_{-CTX-M} gene (247bp). 1.5% agarose gel at 7 volts/cm for 1 hour. Lane 1, 2, 4, 5, 7, 8: positive for *bla*_{-CTX-M} gene, lane 3, 7 were negative, M: 100-bp DNA marker

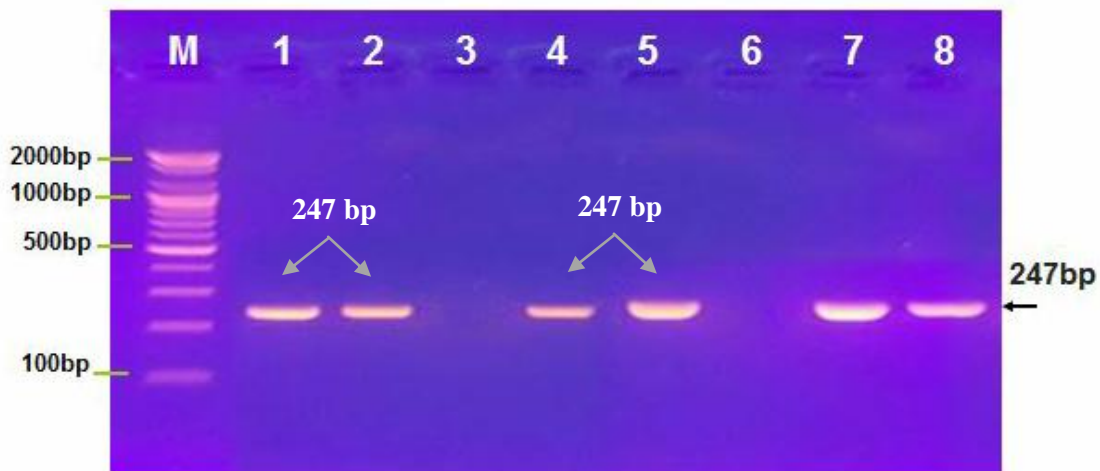


Fig.2 Agarose gel electrophoresis of PCR products of *bla*_{TEM} gene (531bp). 1.5% agarose gel at 7 volts/cm for 1 hour. Lane 1, 3, 5, 6, 7, 9, 10: positive for *bla*_{TEM} gene, lane 2, 4, 8 were negative, M: 100-bp DNA marker

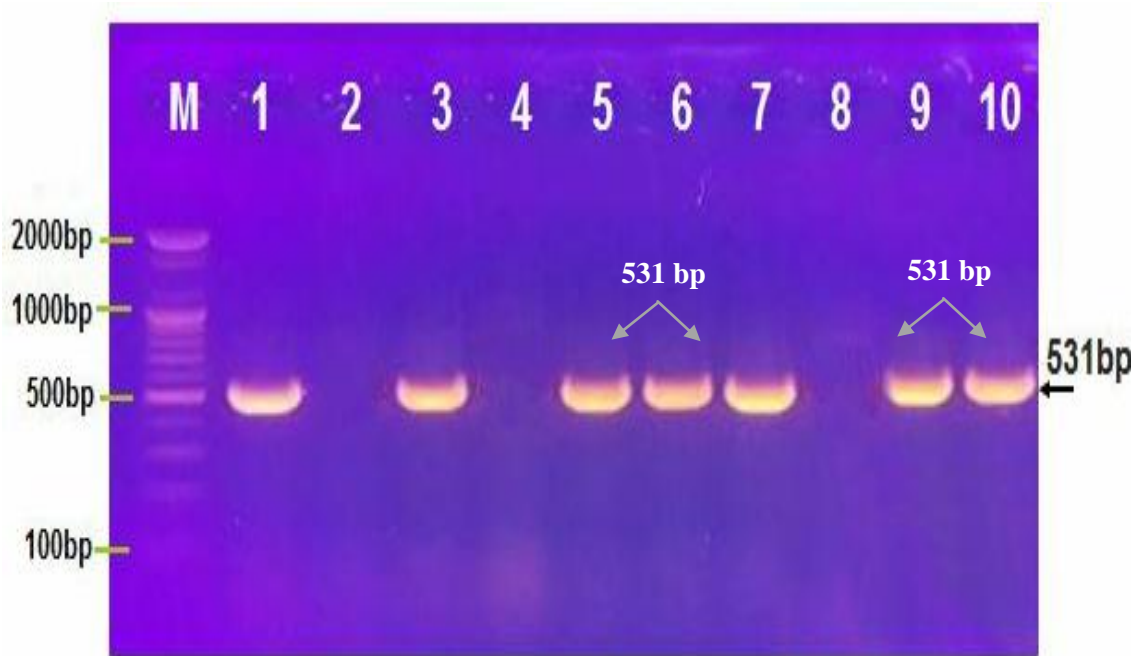


Fig.3 Agarose gel electrophoresis of PCR products of *bla*_{SHV} gene (410bp). 1.5% agarose gel at 7 volts/cm for 1 hour. Lane 1, 3, 4, 5, 6, 7, 9, 10: positive for *bla*_{SHV} gene, lane 2, 8 were negative, M: 100-bp DNA marker

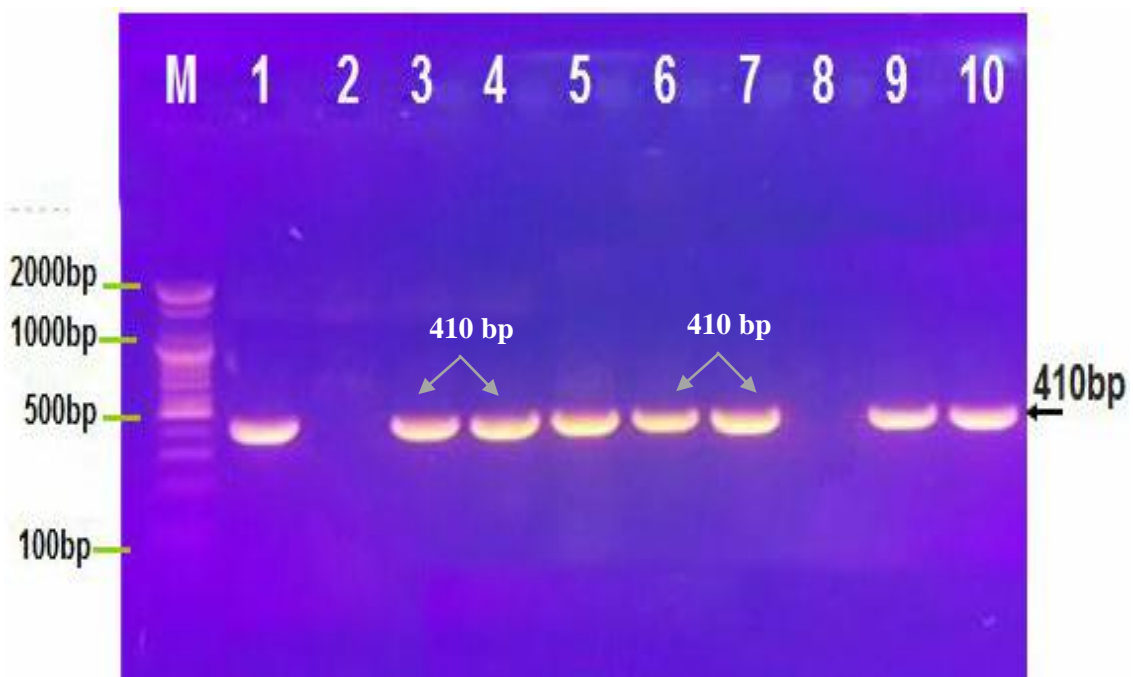
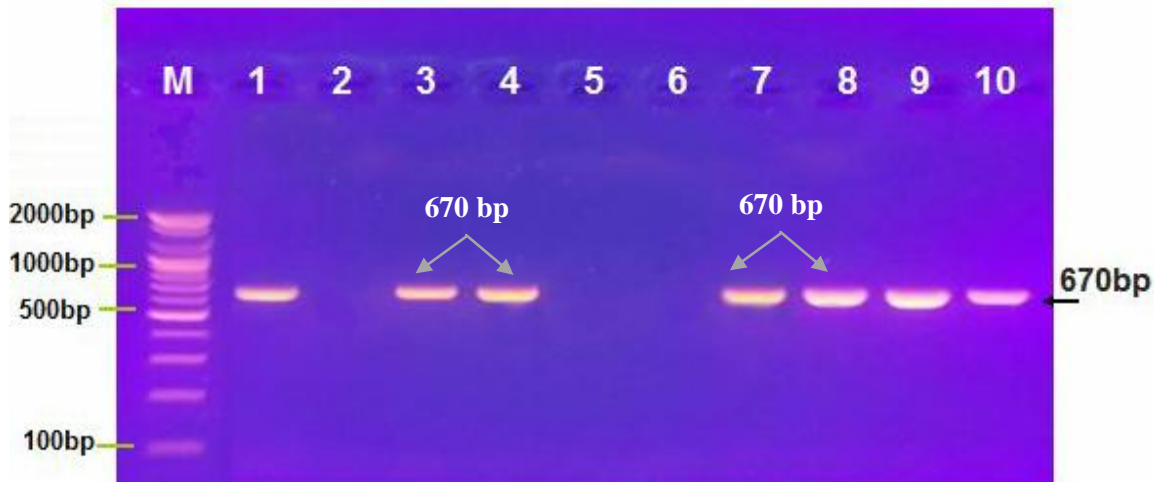


Fig.4 Agarose gel electrophoresis of PCR products of *bla*_{-AmpC} gene (670bp). 1.5% agarose gel at 7 volts/cm for 1 hour. Lane 1, 3, 4, 7, 8, 9, 10: positive for *bla*_{-AmpC} gene, lane 2, 8 were negative, M: 100-bp DNA marker



While *bla*_{TEM} was present in 66.7% and 59.1% in burns and wounds respectively. This fact poses a serious threat to patients as these genes products mediate resistances to a wide range of antibiotics.

To avoid introduction of inappropriate antimicrobial therapy to treatment ESBL-producing bacteria; therefore, by screening those genes frequently when an ESBL is suspected in *P. aeruginosa*, can lead to the use appropriate antibiotics in treatment and help to improve the outcome of infections (Chen *et al.*, 2015)

The prevalence of chromosomal gene "*bla*_{AmpC}" in this study was 53.3% and 50% in burns and wounds respectively, as near as proportion reported by Rafiee *R et al.*, 2014.

They reported the presence in 31 isolates (60.8%), reflecting other difficulties in the treatment of patients infected with ESBL-producing *P. aeruginosa* by decreasing imipenem susceptibility which is a resistance mechanism in *P. aeruginosa*, by corporate AmpC β -lactamases with expanded-spectrum (Rafiee *et al.*, 2014). The high prevalence of ESBLs genes in Diwanya province/Iraq; is an establish fact which need further studies for

detecting resistance genes of ESBL-producing strains of *P. aeruginosa* by molecular methods simultaneous with clinical characteristics of patients may help in reducing failure in the clinical treatment of multidrug resistant pathogenic bacteria.

Disclosure Statement

The authors have no conflicts of interests to declare.

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