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MOLECULAR IDENTIFICATION, SUSCEPTIBILITY PATTERN, AND DETECTION OF SOME VIRULENCE GENES IN *PSEUDOMONAS AERUGINOSA* ISOLATED FROM BURN PATIENTS

Mustafa Dhannoon Younus Ali* and Zirak F. A. Abdulrahman

Department of Biology, College of Education, Salahaddin University Erbil, Iraq.

Abstract

In this study, from September 2018 to January 2019 a total of 150 samples were collected of burns from patients admitted to the West Erbil Emergence Hospital. By using cultural, morphological features, biochemical testing and Vitek's 2 compact systems, 40 isolates of *P. aeruginosa* have been identified. Also Po1ymerase Chain Reaction (PCR) technique performed, through amplification of *16SrDNA and rpoB genes*. The PCR product on gel electrophoresis was 956bp for *16SrDNA* and 759 bp for *rpoB* which confirm that the isolates were *P. aeruginosa*. *P. aeruginosa* produce various colors, including blue / green, and yellow / green as demonstrated by pigments production results. The susceptibility of *P. aeruginosa* isolates to various antibiotics was investigated. Impenim was the most effective antimicrobial agents against *P. aerugionsa* isolates, and most of isolates showed high resistance degree to Ampicillin 100%, Chloramphenicol 100%, amoxicillin-clavulanic acid 100%, Cefotaxime 100% and Penicillin 100% while for Aztreonam 32.5%, Meropenem 42.5%, Tobramycin 45%, Gentamycin 45%, Amikacin 45%, Ciprofloxacillin 62.5%, ceftazidime 67.5,% Tetracycline 80%. All *P. aerugionsa* isolates were screened for their ability to produce Extended spectrum Beta lactamase, out of 40 of *P. aeruginosa* isolates 26(60%) were found to produce ESBL using double disc synergy method. All *Psudomonas aeruginosa* isolates are screened using polymerase chain reaction (PCR) check for the presence of (phzM and exoS) on genomic DNA. The findings have shown that 37(92.5%) of the isolates harbored *phzM* and 34(85%) of isolates harbored *exoS* as a virulence genes.

Key words: *Pseudomona aeruginosa*, antibiotic resistance, and virulence genes

Introduction

P. aeruginosa is a bacterium that causes nosocomial pathogens causing complex disease syndromes (Buhl et al., 2015). It is found that one of the most serious causes of burn wound infections (MR and Hajia, 2012). The skin of human is the principal protective layer of the body's tissues and May contribe to damage and destruct ion of bacteria transmitted to the internal blood tissue, which is rich in proteins and to bacterial infections and which may lead to the development and destruction of these tissues by means of blood (Mahzounieh *et al.,* 2014). Based on its severity (1st, 2nd and 3rd grade burns), classification of burns may be achieved. A different form of inhalation injury is commonly referred to as respiratory fumes and breathing smoke (Andrei *et al.,* 2018). Patients with severe burning wounds are vulnerable to bacterial infections because the skin barrier is physically broken, which allow infections of colonizing organisms in the sweat and hair follicles. Gram-negative bacteria, specifically *Acinetobacte*r, *P.aeruginosa*, and *Klebsiella pneumoniae* are responsible for the majority of burn patient infections. In patients with burned wounds, *P. aeruginosa* is a major problem in hospital because it is transmitted by the patient (Yali *et al.,* 2014). Such diseases also lead to significant morbidity and death (Smith *et al.,* 2014). For patients with (CF), endocarditis, skin wounds or artificial implants, the bacterium may also cause intense life threatening infections (Guragain *et al.,* 2016). Nevertheless, for many experiments, it has been protracted and ineffective and due to their infection's gravity, it has been shown that a fast and sensible technique is required to detect pathogenic *P.aeruginosa* DNA-based techniques such as the (PCR) in an early method (Hassan and Abdullah, 2018). Many of these approaches for defining *P. aeruginosa* have been extended to PCR (McCarthy *et al.,* 2018). Even so, in the last years, treatment of infections caused by these

Author for correspondence : E-mail : mustafa.thanoon@yahoo.com

bacteria has proved difficult because of their resistance to different types of antibiotics and results in increased death rates (Fair and Tor, 2014). The isolates in hospitals are typically more antimicrobial than environmental samples and are more important causes of opportunistic infection of patients (Shilba *et al.,* 2015). *The P. aeruginosa* is responsible in terms of sepsis, cystic fibrosis, burn and bleeding, etc. for approximately 10 – 20% of nosocomial infections. In intensive treatment units (ICUs) (Noha, 2015). Burn centers have defined the bacteriological profile in their hospital to guide observable treatment to provide the right drug at the right time (Bayram *et al.,* 2013). *P. aeruginosa* develops resistance by different mechanisms, including MDR, efflux pumps, biofilm formation (BF), the extend β-lactama development (ESBL) and enzymes altering aminoglycosides (Mahmoud AB, 2013). The intrinsic expression of virulence genes is unique and contributes to a variable rate of pathogens in infected humans (Ahmad *et al.,* 2018). Such infections occur mainly due to drug resistance patterns growth, formation of biofilms and the synthesis of virulence factor (Balasubramanian *et al.,* 2013). Biofilm has strong immune system and antibiotics resistance, which is a source of chronic and persistent infection (Wei and Ma, 2013). A strong candidate for developing *P aeruginosa* vaccine was found in their external membrane protein (OprI and OprL) that activate a patient's immune system (Ingle *et al.,* 2017). The strong association among virulence genes and infection origin may help to control these shared infection (Ahmad *et al.,* 2018). Querum sensing has been the strongest method for controlling your expression and will therefore boost therapeutic success in the future, targeting certain primary regulators (Balasubramanian *et al.,* 2013). Therefore the aim of this study is isolation and identification of *P.aeruginosa* from burn patients, determination of antimicrobial susceptibility patterns, the occurrence of ESBL phenotypically in these isolates, and molecular detection of some virulence genes of *P. aeruginosa* by PCR screening.

Meterials and Methods

Patients and specimens collection

One hundred and fifty samples were collected from patients admitted to West Erbil Emergency Hospital, between September 2018 and January 2019. Following collection, each sample was cultured on different culture media and *P. aeruginosa* was identified by cultural characters, biochemical methods, and molecular assay.

Antibiotic sensitivity test

Mueller-Hinton agar was used as growth media to investigate the effects of different antibiotics on *P. aeruginosa* isolates. The Kirby Bauer method was used to determine antibiotic resistance patterns of isolates; Adjustment of the bacterial inoculates to the Clinical and Laboratory Standards Institute of 0.5 McFarland standards (CLSI, 2011). A sterile cotton swab was used to disperse the sample inoculum to Mullur-Hinton Agar. The antimicrobial products tested, including: Impenim (IPM), ceftazidime (CAZ), Tetracycline (TE), Ampicillin (AM), Chloramphenicol (C), amoxicillin-clavulanic acid (AMC), Amikacin (AK), Cefotaxime (CTX), Gentamycin (CN), Ciprofloxacillin (CIP), Penicillin (P), Aztreonam (ATM), Meropenem (MEM), Tobramycin (TOB) Muller Hinton inoculated agar was placed aseptically and incubated overnight. The zones of inhibition were interpreted and measured (CLSI, 2011).

Color production by *P.aeruginosa* isolates

Bacterial *P.aeruginosa* samples have been inoculated on Cetrimide agar, incubated for 18-24 hours by streaking method at 37°C, and then the pigment production was examined.

ESBL Detection (Extended spectrum beta lactamase) activity

Forty *P. aeruginosa* isolates were screened for ESBL production using double-scale synergy methods; the checked inoculums were dispersed onto the Muller-Hinton agar using sterile cotton swabs (adjusted at 0.5 McFarland turbidity). The central disks of amoxicillin-clavulanic acid AMC were kept around CTX, CAZ and ATM at a distance of between 16 mm and 20 mm (center to center) from the amoxicillin-clavulanicacid disk. Overnight at 37 °C the plate was incubated (PA, 2015).

Polymerase chain reaction assay (PCR) DNA extraction

Two hundred micro1iter of overnight growth, l.5 is separated from supernatant in the 2 m1 micro- centrifugal tube extracted for 30 seconds at l3000 rpm. The pellet has been dissolved in 200 µl TL buffer, then removed and fully mixed with 20 µl proteinase k so1utions to achieve a uniform suspension. The sample has been incubated in the water bath at 56°C for ten minutes until the cells have been completely lysed. 200 µl of GB buffer applied to the specimens, then by vortexing mixed thickly for approximately l5 sec up to a uniform mixture and then incubated for l0 minutes at 56°C. Then 200 µl of absolute ethanol is added and pipetted or vortexes. The lysate transfered carefully without wetting the rim into the spin column reservoir For 1 minute at l0,000 rpm, and the column centrifuged the collection tube then discharged containing the flow- through solution a new 2 ml tube has

been placed with the GeNet Bio genomic DNA purification co1umn. 500 µl of GW1 buffer was added then centrifuged for l min at l0,000 rpm, the flow-through discarded and the purification column placed back into the collection tube, 500 µl of GW 2 was added to the GeNet Bio genomic DNA purification co1umn, Centrifuged for l min at l0,000 rpm. Then after centrifuging the tube, remove the f1ow-through and reassemble the spin co1umn with its collection tube, again, centrifuge at l2000rpm to l2 minutes to extract ethanol completely and check that the droplet is not attached at the bottom of the tube. Move l.5 ml of the spin co1umn to a new tube to do the elution. Add 200 µl of elution buffer to the center of the GeNet Bio kit genomic for DNA purification co1umn membrane, genomic DNA elution. Incubated at room temperature for 1 min and centrifuged for l minute at 10,000 rpm. The purified DNA was immediately removed or deposited at 20°C in downstream applications.

PCR amplification

PCR was used for the identification of the *16SrDNA* in genomes of *P. aeruginosa* intrinsic to these bacteria with amplicone length 956 bp and *rpoB* 759 bp amplicone genomes, The *16SrDNA* used were forward 5'- GGG GGA TCT TCG GAC CTCA -3' and reverse:5'- TCC TTA GAG TGC CCA CCC G -3', and the *rpoB* primers were: forward 5'- CAG TTC ATG GAC CAG AAC AAC CCG-3' and reverse:5'- ACG CTG GTT GAT GCA GGT GTT C -3', the program of PCR for *16SrDNA* 94°C for 10 min, then 35 cycles at 94°C for 1 minute, for 1 minute at 50°C, and at 72°C for 1 min, followed by a final extension at 72°C for 12 minutes (Megahed *et al.,* 2015). and PCR program for *rpoB* gene was 94°C for 3 minutes, and then 35 cycles at 94°C for 1 minute, at 58°C for 1 minute, and at 72°C for 2 min, this followed by 2 minutes of final extension at 72°C for (Comoe koffi donation benie., 2017). PCR was performed in a 50µl of reaction volume. Master Mix tube contains 25 µl total of forward and revers Primers with 5 µl for each, and DNA template 5 µl, lastly sterile deionized water 10 µl.

Detection of *phzM* and *exoS* genes in *P. aeruginosa*

PCR also was used for detection of *phzM and exoS* genes in *pseudomonas aeruginosa* genome; and the size of its amplicon was 363 bp and 504 bp. forward primer used for *phzM* 5'- GCC TTC CAT TGA GAT CCC CAG -3' and reverse 5'- CGA GAT GGT TCG CTC GAT CA -3'. And for *exoS* the primer used was forward 5'- CTT GAA GGG ACT CGA CAA GG-3' and reverse 5'- TTC AGG TCC GCG TAG TGA AT-3'. Program of the PCR for *phzM* 35 cycles of amplification were followed after initial denaturation (94°C for three minutes). Each cycle consisted of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. A final extension step (72°C for 5 minutes) completed the amplification (Comoe koffi donation benie., 2017). And the program for the *exoS* was Primary denaturation 94°C for 5 min followed by 35 cycles of amplification, Second denaturation 94°C for 45 seconds, Annealing 58°C for 1 minute, Extension 72°C for 1 minute, and Final extension 72°C for 7 minutes (Comoe koffi donation benie., 2017). PCR conducted for both genes were performed in a 50µl of reaction volume. Master Mix tube contain 25 µl, forward and reverse primers with 5 µl foe each primer, DNA template 3 µl, and lastly sterile (D. W) deionized water 12 µl.

A garose gel electrophoresis

To perform gel electrophoresis a method of (Judelson, 2001) was followed with minor modifications. Adding 1.2 g agarose to 100 ml 1x TBE buffer was used as an agarose gel, the mixture melted for 1-2 min in the microwave oven or until it was apparent and fully dissolved. Left to cool at about 50°C, 10µl of primary safe dye was carefully added to the agarose solution and thoroughly mixed with a gentle swirling. The edges of tray sealed with the tape and the proper comb inserted into the tray. The agarose gradually poured in the tray and any bubbles with a disposable tip pushed away to the side at room temperature, the agarose solidified (15-30 min). The tape was removed from the tray and then the tray was placed in the electrophoresis tank. The tank filled with more TBE buffers, so that the gel is completely under buffer. PCR product loaded into the wells (15µL) with loading buffer. Depending on the size of the PCR sample the first well (5µl) (1 kb or 100 bp) was used. The gel runs for 50 minutes at 100 V. Finally, the UV transilluminator and gel photographed.

Results and Discussion

Collection of *P. aeruginosa* isolates

A series of confirming tests were conducted to verify that all bacterial isolates reclaimed belong to species of *P. aerugionsa*. This smear preparation of bacterial cells are gram negative rods, mobile, non-spore-forming, arranged in single or short chains. The colonies were thin, rough or smooth on solid media with flat edges and high appearance, but some were mucoid in aspect, biochemical testing was negative for Indole, TSI, positive for oxidase and Catalase, positive for citrate, positive for urease (slowly hydrolysis the urea), and are probably P.aeruginosa, Both isolates have also been classified using Vitek 2's compact bacterium ID method, and tests have

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Target gene	Primer sequences	Amplified segment (bp)	Primary denaturation	Second denaturation	Annealing	Extension	Final extension	Reference
16SrDNA	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	94 C10 min.	94 C1 min.	50 C1 min.	72 C1 min.	72 C12 min.	(Megahed et al., 2015)
rpoB	CAGTTCATGGACCAGAACAACCCG ACGCTGGTTGATGCAGGTGTTC	759	94 C3 min.	94 C1 min.	58 C1 min.	72 C2 min.	72 C2 min.	(Comoe koffi donation benie., 2017)
exoS	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504	94 C5 min.	94 C45 sec.	58 C1 min.	72 C1 min.	72 C7 min.	(Comoe koffi donation benie., 2017)
phzM	GCCTTCCATTGAGATCCCCAG CGAGATGGTTCGCTCGATCA	363	94 C3 min.	94 C1 min.	55 C1 min.	72 C1 min.	72 C5 min.	(Finnan et al., 2004)

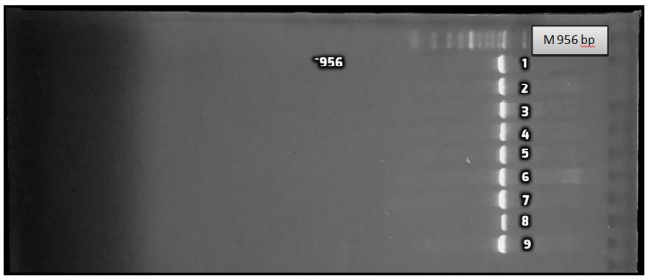

Fig. 1: PCR amplification of *16SrDNA identification* gene of *P. aeruginosa*, lanes 1, 2, 3, ...9 represent amplified product (956 bp) of *P. aeruginosa* isolates, ladder (M) 956 bp.

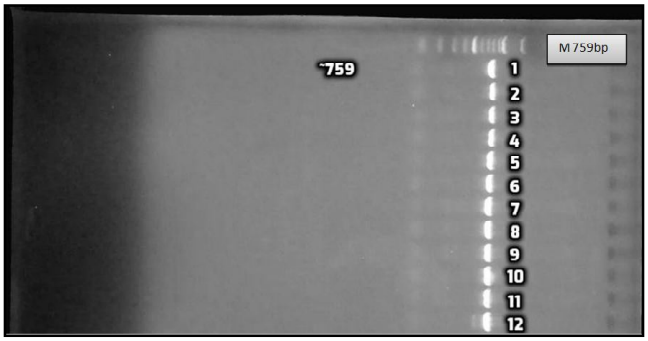

Fig. 2: PCR amplification of *rpoB identification* gene of *P. aeruginosa*, lanes 1, 2, 3, ...12 represent amplified product (759 bp) of *P. aeruginosa* isolates, ladder (M) 759 bp.

shown that both recovered isolates belong to *P.aeruginosa* species, as indicated in previous observations (Abro *et al.,* 2009).

Molecular Identification of *Pseudomonas aeruginosa*

The detection of *16SrDNA and RpoB* genes in extracted DNA for all isolates, using the PCR technique by detecting the *16SrDNA* and *rpoB* genes, was carried out to verify the identification of *P. aeruginosa* species by the molecular process. All 40 *P.aeruginosa* isolates are PCR-positive for *16SrDNA* and *rpoB*, and this is shown, in figures (1 and 2), by *16SrDNA* (956bp) and *rpoB* (759bp) amplified genes. The results of the previous report confirm the findings, which indicate that *16SrDNA* and *rpoB* are ubiquitous. 16SrDNA and rpoB were used to classify *P. aeruginosa* (Megahed *et al.,* 2015) and (Tayeb *et al.,* 2005). These results show that these two genes can be used to classify *P. aeruginosa* as a simple and reliable way. In all isolates of *P. aeruginosa*, *16SrDNA* and *rpoB* are found. The high molecular identification rate has indicated that genomic trials were important to confirmed *P. aeruginosa's* exact taxonomic role. The success of the *rpoB* gene on the identification of *P. aeruginosa* strains may be supported by an observation of the *rpoB* gene (Tayeb *et al.,* 2005) which discriminates between species very similar to

Pseudomonas.

Antibiotic sensitivity profile of *Pseudomonas aeruginosa* isolates:-

forty *P. aeruginosa* isolates were screened for their resistance to (15) widely used antibiotics in which are (Amikacin, amoxicillin-clavulanic acid, Ampicillin, Cefotaxime, Penicillin, Ciprofloxacin, Chloramphenicol, Gentamcin, Imipenem, Meropenem, Tetracyclin, Ceftazidime, Aztreonam, Tobramycin). Table 2 illustrates that all isolates vary in their response to the used antimicrobial agents, the most effective antibiotic was Imipenem and the resistance percentage was (%0). The resistance percentage for Ciprofloxacillin 62.5%, for Gentamycin 45%, for Amikacin 45%, Meropenem 42.5%, Aztreonam 32.5%, for Tetracyclin 80%, Tobramycin 45%, Ceftazidime 67.5%, while the highest percentage of resistance (100%) was to (Chloramphenicol, Ampicillin, cefotaxime, Penicillin, AMC amoxicillin-clavulanic acid). The spread of resistant in *P. aeruginosa* over recent decades is unbelievably increased which restricts the choice of the therapeutic choice for the treatment of this microorganism's infection. *P. aeruginosa* isolates are increasingly resistant to more anti-microbial substances in all countries. (Olayinka *et al.,* 2009) reported that 20% of *P. aeruginosa* isolated from clinical sample obtained from the surgical units Ahmadu Bello university teaching hospital in Nigeria were sensitive to imipenem which is in a good agreement with our results, Imipenem and meronem are ß- lactam antibiotics that have broad-spectrum activity against Gram-negative and Gram-positive bacteria (Joly-Guillou *et al.,* 2010). All bacterial isolates displayed a low resistance and the majority of Enterobacteriaceae isolates showed no resistance. It might be because they are reserve medicines and they are used as the last option in our hospital environment for multidrug-resistant bacteria which differ with our result. In the case of (Fattma A. Ali, 2017), 98% of *p. aeruginosa's* isolates resist Amkacine 96% for Cephotaxime, 80% for Rifampicin, 70% for Ampicillin, 70% for augment and 60% for Dooxycycline, which is against our performance, respectively. Resistance by *P. aeruginosa* can both be due to inducibly beta-lactamases, which can make cephalosporin of broad-spectrum inactive and to beta-lactamases mediated by plasmid, which can lead to several peniciellins and ancient cephalosporin becoming resistant (Baird, 1996). Mechanisms of aminoglycoside resistance in clinical isolates are usually controlled by enzymatic antibiotic inactivation since nine different enzymes that are capable of catalyzing phosphorylation, acetylation, aminoglycosides coradenylylation in bacteria have now been described (Pollack, 2000). The development of the *P. aeruginosa* multi-resistant and its antibiotics mechanisms involves decreased cell permeability, efflux pumps, and changes in target enzymes and antibiotics inactivation (Lambert, 2002).

Colour production by *P.aeruginosa* isolates

Isolates are grown on a single medium for the production of pigments. When they were cultivated on selective media at 37°C, the colors created by isolates were noted. All isolates were cultivated with cetrimide agar, a medium that stimulates pyocyanin production. Such pigments are common of *P.aeruginosa*. Isolates produced yellow / green for the production of fluorescein, blue / green for the production of pyocyanin. Pigment development is a phenotypic contributing factor in the *P. aeruginosa* group (Abro *et al.,* 2009). The medium of growth has the greatest impact on pigment production. Different media are required for the production of pseudomonads of different pigments. The pigment serves as a pro inflammatory agent for phagocytes, destroys human nasal cilia's normal function and impedes human and lymphocytic epidermis. This may be due to electron transport inhibition. Antifungal properties that provide *P. aeruginosa* with an obvious selective advantage in their natural environment have been identified (Loveday *et al.,* 2014). It also induces rapid apoptosis of human neutrophils (Allen *et al.,* 2005), directly oxidises glutathione and decreases its levels in airway epithelial cells (O'Malley *et al.,* 2004).

ESBL production in P. *aeruginosa*

Twenty six (60%) isolates from 40 samples were found to be positively ESBL, compared to 14 negatively ESBL. As stated by (Amutha *et al.,* 2009), the ESBL mediated resistance in *P.aeruginosa* was (25%). (Kaur and Singh, 2018) Have also revealed that ESBL produces from Various Clinical Samples of *P. aeruginosa* was (17.7%). This study shows a prevalence of *P. aeruginosa* generating ESBL that is significant as such strains can often cause pediatric outages and cause increased morbidity and mortality and reduce therapeutical choices because of the high degree of multidrug resistance in patients suffering from underlying illnesses. Patients with predispositive factors such as hospital stay, seriousness of illness, reduced immunity and urinary catheterization can be the highest rate of ESBL-produced bacteria (Mirsalehian *et al.,* 2008).

Detection of phzM and exoS genes in *P. aeruginosa*:

This study was conducted for 40 isolates of *P. aeruginosa*, which were recovered from burn patients for molecular detection of *PhzM* and *exoS* genes in this

Table 2: Resistance of *P. aeruginosa* to antibiotics

Antibi-otics	No. of Isolates	% of Resistant	% of sensitive
AMC	40	100%	0
AM	40	100%	0
CAZ	40	67.5%	36.5%
AK	40	45%	55%
CIP	40	62.5%	37.5%
ATM	40	32.5%	67.5%
MEM	40	42.5%	57.5%
TOB	40	45%	55%
IMP	40	0	100%
C	40	100%	0
CN	40	45%	55%
TE	40	80%	20%
CTX	40	100%	0
P	40	100%	0

study. The results showed that 37(92.5%) of the *phzM*-harbored isolates and 34(85%) of the isolates carry *exoS* as shown in (Figs. 3 and 4). These results are consistent with the findings of Mavrodi *et al.,* (year) who stated that PCA was converted to pyocyanine by two different enzymes, *PhzM* and PhzS, and *PhzS* was involved in biosynthesis as well as pyocyanine development. (Mavrodi *et al.,* 2001). Thus, appearance of yellow pigment in *P. aeruginosa* is a result of the presence of *PhzS*, and appearance of green pigment in *P. aeruginosa* strains represents the presence of both *PhzM* and *PhzS* proteins. Feltman and others (Feltman *et al.,* 2001) recorded that of 115 *P. aeruginosa* isolates, 82 contained *exoS* and recent studies found the simultaneous detection of exoS+ExoU in clinical and environmental *P. aerugino* isolates to be frequent. (Finnan *et al.,* 2004) found 75% of these two genes were spread in *P.aeruginosa* isolated from various clinical sources. Results have shown that the prevalence of *P. aeruginosa exoS* virulence gene in Burn injury infections was higher than CF. In the bacteria isolated from children with CF it has been found that the number of *toxA, lasB* and *exoS* was significantly high. In France study which has isolated 162 *P. aeruginosa* from various body parts infections indicated a greater prevalence of *exoS* and *nan1* than the other genes of

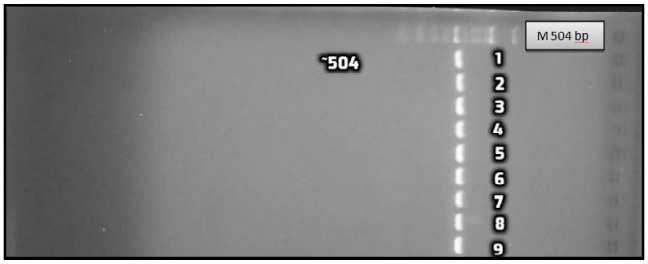

Fig. 3: PCR amplification of *exoS virulence* gene of *P. aeruginosa*, lanes 1, 2, 3, ...9 represent amplified product (504 bp) of *P. aeruginosa* isolates, ladder (M) 504 bp.

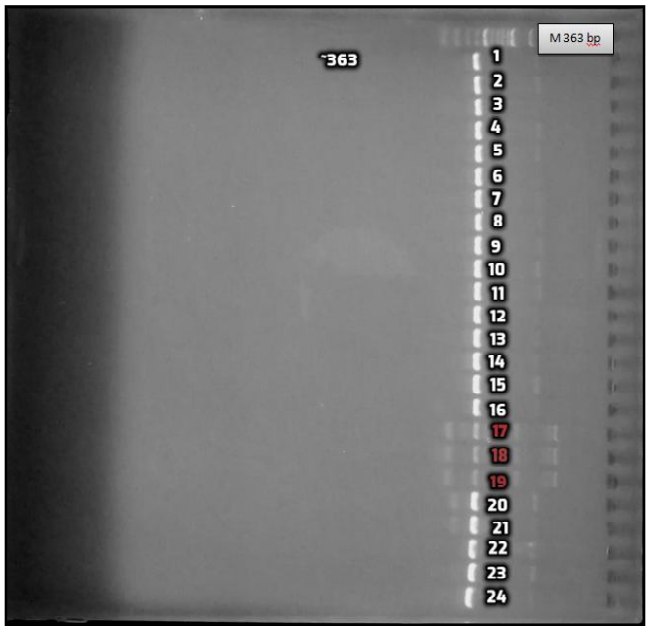

Fig. 4: PCR amplification of *phzM virulence* gene of *P. aeruginosa*, lanes 1, 2, 3, ...24 (except 17, 18, 19) represent amplified product (363 bp) of *P. aeruginosa* isolates, ladder (M) 363 bp.

virulence. *ExoS* has been shown to play an important role in CF infection; this virulence factor impairs lung phagocytosis which exacerbates the infections (Sanders and Goss, 2013).

Conclusion

The results show that 100% of P. aeruginosa isolates possessed 16sr DNA and RpoB as identity genes for burn and wound infections, while 92.5% of those contained phzM genes and 85% exoS as virulence genes. In recognition of the significance of the rapid and early detection by biochemical methods of pathogenic strains of any bacteria, PCR for Multiple Virus Genes (phzM and exoS) is suggested for the identification of the *P. aeruginosa* pathogenic strains. This test can be used for the testing of some antimicrobial infections.

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