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Molecular Detection of *fur* and Iron Responsive Genes in Local Isolates of *Pseudomonas aeruginosa* from Wounds and Burns

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Abstract: Fourty samples were obtained from burns and wounds, from various ages. specimens were collected through the period extending from February to December 2022, from patients were admitted in AL-kindy hospital,Alnumaan teaching hospital,Ibn-albaladi for women and children and Imam Ali hospital in Baghdad. Identification of isolates were performed by biochemical and cultural tests, the results revealed that all isolates were *P. aeruginosa*. Conventional PCR (Polymerase Chain Reactin) was also used to confirm these isolates as *P. aeruginosa* by detection of *16 SrRNA* but the results showed that this gene was located in only 30(75%) out of 40 biochemically *P. aeruginosa isolates*. PCR was also used to screen *fur* gene and iron responsive genes which are (*pvd ,pch ,exoS and exoA) of the thirty* isolates of *P.aeruginosa and* results revealed that *exoS* gene was located in only 14(46.6%) out of 40 isolates, while for *pvd* gene results revealed only 21(70%) of isolates were consist of this gene out of 30 *P. aeruginosa* isolates, for *pch* gene 15(50%) of isolates were consist of this gene out of 30 *P. aeruginosa* isolates, 20 (66.6%) were appeared to have for *exoA*gene out of 30 *P. aeruginosa* and 27(90%) of isolates were positive for *fur* gene. The source of these isolates which was burn swab n=20 (66.6%) and wound swab n =10 (33.3%).

Key words: *P. aeruginosa* ,*fur* , *exo* A , *pvd*,*pch* , *exo*s , Burn wound infection.

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Introduction

Pseudomonas aeruginosa is a type of Gram-negative bacillus that lacks the ability to produce spores and it is motile by the presence of one or two polar flagella.The organism in question is classified as a strictly aerobic aerobe(1). The organism's identity is frequently ascertained by looking at its colony morphology, determining whether it is oxidase positive, noticing the presence of distinctive colors, and measuring its capacity to grow at 42°C(2). *Pseudomonas aeruginosa* is an aerobic, Gram-negative rod can infect weakened immune compromised pateints(3). *P. aeruginosa* exhibits a variety of behaviors, including Quorum sensing, biofilm formation, and virulence factors, supported by intricate molecular pathways and genotypic alterations(4). Many acute and chronic infectious diseases can be caused by *P.aeruginosa* with a wide range of symptoms such as inflammation and sepsis specificaly in immumocompromised pateints those with burn infections and pateints with HIV and cancer (5).Nosocomial infection is a major concern for burn patients. Infection is a major source of morbidity and mortality in burn patients

in hospitals, so nosocomial infection become the most common and more dengrous in patients with burns because of their weakened state and the nature of the damage (6). *P. aeruginosa* is highly pathogenic and requires iron as important metal which enable bacteria to cause disease.Many virulence factors in *P.aeruginosa* play an important role in pathogenicity these factors are iron responsive genes as (*pvd,pch,exoS* and *exoA*) in addition *fur* gene which act as a repressor for iron responsive genes by producing FUR protein.Under iron replete conditions *P.aeruginosa fur* gene up-regulate producing FUR protein which bind to iron and be able to bind to DNA and inhibit the expression of their target genes. However, in the presence of low metal concentrations, a conformational change occurs, preventing DNA binding and relieving the repression, so iron act as a co-pressor bindig with fur which act as apo-pressor to regulate iron responsive genes (7). *pvd gene* responsible of pyoverdine a sidrophore with high affinity to iron. This compound have the ability to scavenge and help get iron into the bacteria's internal compartment(8).

The *pch* gene responsible of pyochelin ,the second kind of sidrophores that is categorized as a salicylate-based siderophore and has a relatively low affinity for iron (9).*exoS* gene responsible of ExoS enzyme, is a crucial component of *P. aeruginosa* in vivo pathogenicity that allows it to evade phagocytosis and ultimately kill the host cell (10), *exoA* gene responsible of exotoxin A functions as an ADP-ribosyl transferase (11),is an important virulence factor for lung, corneal, and septicemia infections (12).

Materials and Methods

This research was aimed to study the relationship between fur gene and iron responsive genes under availability condition and it`s role in pathogenicity of bacteria in order to obtain a pathway dependent on iron concentration to inhibit the ability of bacteria to cause disease.This study was performed between February to December 2022, which included 40 clinical isolates from burns n= (26) and wounds n=(14) of different ages.Samples collected from patients were admitted in Imam Ali hospital, Alnumaan teaching hospital ,AL-kindy hospital and Ibn-albaladi for women and children in Baghdad.

Laboratory tests

Isolation of bacteria

The ability of bacteria to grow on specific culture media and the study of growth charecteristics was considered to identify all isolates as *P.aeruginosa.* MacConkey agar, blood agar and cetrimide were used, then all traits of bacteria on these media were observed like morphology, size and color of bacterial colonies. and the changes caused by the growing colonies on these media showed many changes in colour and PH of media which were studied and their phenotypic characteristics were observed, Oxidase, catalase and IMVc biochemical tests were carried out also.

DNA extraction

Genomic DNA extraction Kit by (Trans) was used to achieve his step of DNA extraction. Bacterial suspension was prepared when isolate was inoculated in Brain heart broth (BHB) media (incubated at 37°C for 24 h), the turbidity was adjusted for obtaining approximately $1x10^9$ CFU/ml, and then 1ml is transferred of suspension to1.5 ml Eppendorf tube and is centrifuged at 14000 xg for 1 min.

Preparation and Optimization of the Primer

Preparation of Primer

The source of all primers used in this study was newley design according to the a certain program by a company called Macrogen® (Korea). The name, sequence and product size are given in table (1).

Detection *16SrRNA* gene of *Pseudomonas aeruginosa* by using polymerase chain reaction technique

The PCR mixture was prepared from the master mix, size 12.5 µL, 3 µl of DNA sample, 1 µl 10 pmol/µl from each primer, 7.5 µl of free-nuclease water. After mixing the contents of the PCR tubes the contents were adding to the PCR apparatus, the reaction mixture's final volume was 25 µl. Next, 5µl of the duplicate gene was introduced for electrophoresis onto the prepared 1.5 % agarose gel.

Amplification of *16SrRNA* gene

EasyPure®Genomic DNA Extraction Kit (Transgene® China) was employed to get the DNA of 40 isolates from colonies identified as *P. aeruginosa*. Identification of all 40 of *P. aerugenosa* isolates was performed by using specific primer for 16S *rRNA* gene which give the only 30 isolates as *P.aeruginosa.* Company of ladder is a new England biolabs and the type is low molecular weight DNA ladder.

Molecular Detection of *fur* and iron responsive genes using polymerase chain reaction (PCR) technique.

The PCR mixture of *fur* and iron responsive genes (*pvd, pch, exoA and exoS)* were prepared as the similar way which used to prepare the reaction mixture of the *16SrRNA* gene.

Table (1): Primers sequences, length, and PCR product.

Gene	Primer Sequence	Product size (bp)	Annealing Temp(c)	Reference
16SrRNA	F 5` GCCTCATGCCATCAGATGTGC 3` R 5` GCAATATTCCCCACTGCTGCC 3`	158	$60C^0$	Newley designed
exo S	F 5` CTCAACGTCCGCTGGAACTGA 3` R 5` CAACTGCGTCATCCCATGCAA 3`	172	$60C^0$	Newley designed
pvd	F 5` ACCAGCCCGTATTCCTTCGTC 3` R 5` CAGCGCAGGTAGTCGTTGAAC 3`	113	$60C^0$	Newley designed
pch	F 5` TTCCGAATCGCCTACCAGACC 3` R 5` CAGCACGAAGGGATGGATCGT 3`	156	$60C^0$	Newley designed
exo A	F 5` TGCAACCTCGACGATACCTGG 3` R 5` TGATGACCGTGGGCTTGATGT 3`	94	$60C^0$	Newley designed
fur	F-5`-ACCAGCCCGTATTCCTTCGTC-3` R-5`-CAGCGCAGGTAGTCGTTGAAC-3`	113	$60C^0$	Newley designed

Results and discussion

Following the morphology in gram staining, cultural traits, and biochemical features, the isolates were identified. Fourty samples in all were grown on medium MacConkey agar and cetrimide agar. Elevated coloneis with florescent green color grape like odor was appeared on cetrimide agar, while observed as colorless colonies on MacConkey's agar, indicating that it does not ferment lactose, and appeared greenish due to the formation of pigments with color on regular nutrients media. The isolates were showed positive oxidase and catalase test (13). When PCR was performed on all isolates the results showed only 30 (75%) of the 40 biochemically isolated *P. aeruginosa* isolates had *16SrRNA*.

The product of conventional PCR for 40 isolates detected by using gel electrophoresis was showed in Figure(1). The presence of other closely related species may showed the same biochemical results as *P. aeruginosa* but they lack *16SrRNA*, in which case PCR testing were more accurate than traditional biochemical tests (14). When traditional methods was showed that all isolates were *P. aeruginosa*, the findings by PCR was showed that 30 (75%) of the 40 biochemically isolated *P. aeruginosa* isolates had *16SrRNA*. In accordance with this, ten isolates that were identified as *P. aeruginosa* using the conventional approach lacked this gene. These results are partially agreement with results in study by (15) who discovered that 50 (96.1%) of the 52 biochemically isolated *P. aeruginosa* samples had 16SrRNA.

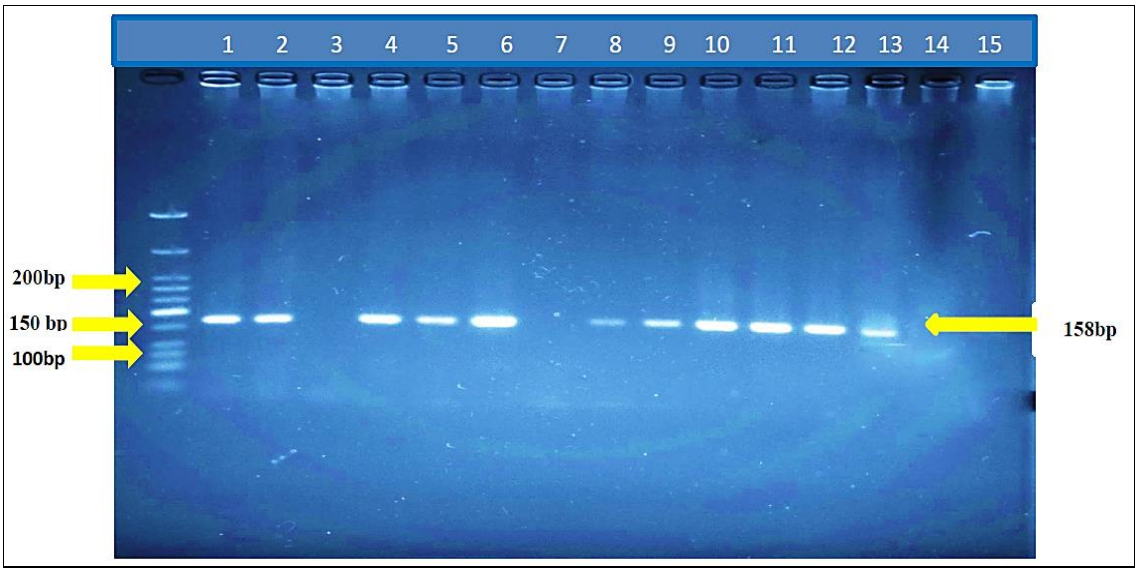

Figure (1): Gel electrophoresis of amplified PCR product of *16SrRNA* Gene (158bp) of *P.aeruginosa* bacterial isolates at(90v.for 70min.) stained with red safe stain and were fractionated on 1.5% agarose gel electrophoresis. M:25bp ladder marker.

Molecular detection of target genes *fur, pvd, pch, exoA and exoS* in *P. aeruginosa* isolates

By using particular primers for each gene, conventional PCR amplification was carried out for *P. aeruginosa* to confirm the presence of target genes, which play major role in regulation of iron acquisition which in turn enhances pathogenicity of *P. aeruginosa*. The product of PCR was then detected using gel electrophoresis.

Out of 30 *P. aeruginosa* isolates, the results indicated that 20 (66%) had an *exo A* gene; the amplified PCR product for *exo A* was electrophoresed on a gel as in Figure (2). This result is in partial agreement with (16), who reported that 10 (83.3%) isolates out of 12 *P. aeruginosa* isolates had the *exo A* gene; however, it disagrees with (17), who reported that the PCR method targeting the *exo A* gene detected 57 (50%) positive samples out of 364 total samples due to the number of isolates. Other Study revealed that the *exo A* gene was located in 4 (57.1%) out of 7 isolates (18); additionally, they agreed with (19) a study which found *exo A* gene located in 96 (76.8%) out of 125 isolates.

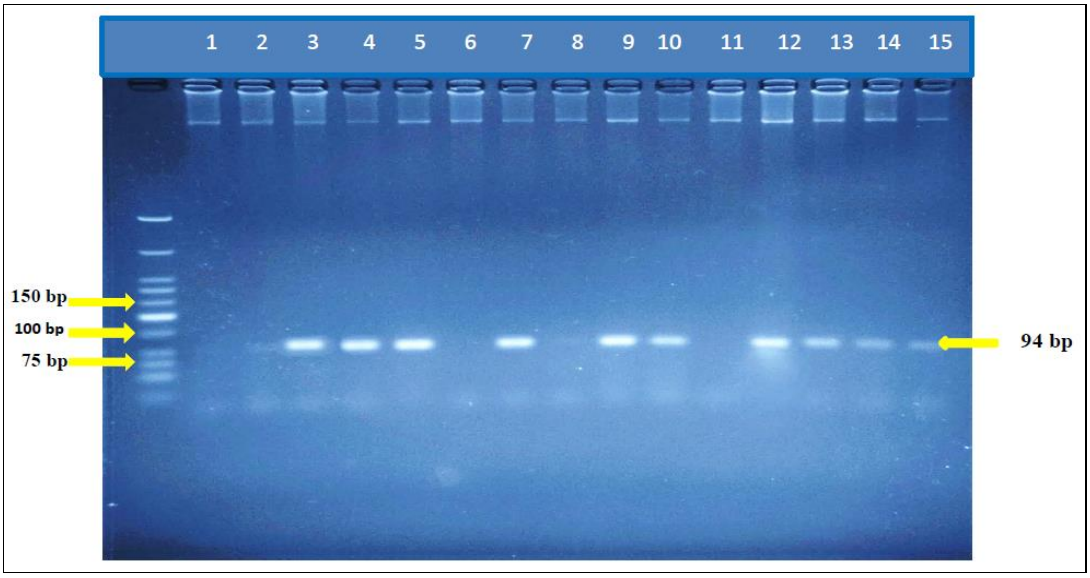

Figure (2): Gel electrophoresis of amplified PCR product of *exoA* Gene (94bp) of *P. aeruginosa* bacterial isolates at (90v.for 70min.) stained with red safe stain and were fractionated on 1.5% agarose gel electrophoresis. M: 25 bp ladder marker.

The *exoS* gene location was also shown by PCR assays to be present in 14 (46.6%) of the 30 isolates; the gel electrophoresis of the amplified PCR product for *exoS* is displayed in figure(3). The findings aline with a study by (20) which found that *exoS* gene was located in 42 (26.3%) out of 160 isolates, and the study by (21) found that the *exoS* gene was located in 56.9 (90.47%) out of 63 isolates, as well as the findings of the study in (22) which found that the *exoS* gene was negative in all isolates, and other study found that *exoS* gene was revealed in 87 (87%) out of 100 isolates (23). These partially in agreement with studies described.

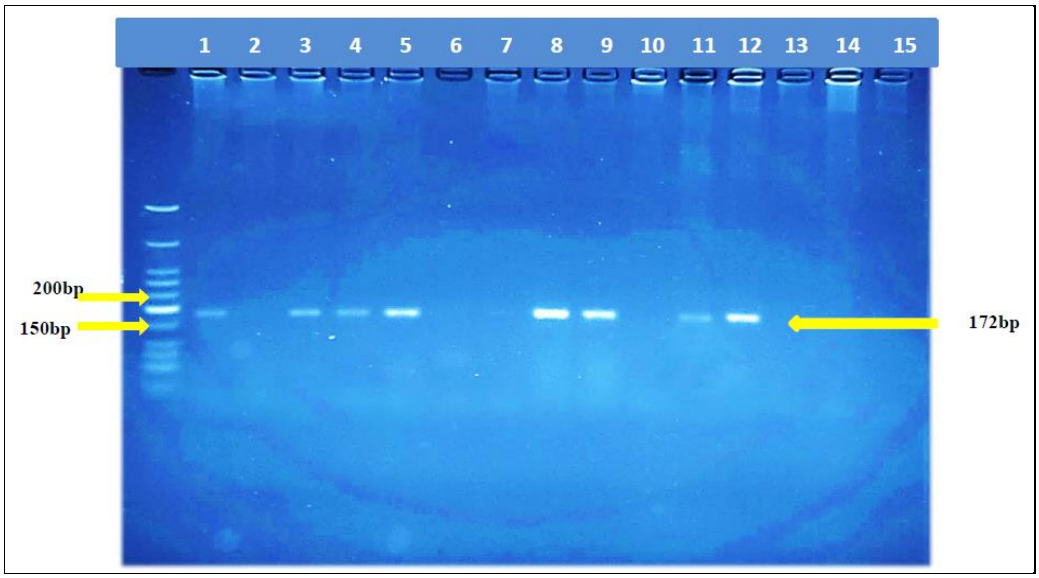

Figure (3): Gel electrophoresis of amplified PCR product of *exoS* Gene (172bp). of *P. aeruginosa* bacterial isolates at(90v.for 70min.) stained with red safe stain and were fractionated on 1.5% agarose gel electrophoresis. M: 25 bp ladder marker.

The results of the *pvd* gene by PCR assays revealed that 21 (70%) out of 30 isolates of *P. aeruginosa* carried this gene. The gel electrophoresis of amplified PCR product for *pvd* showed in figure (4), so this result agrees with (24), the study revealed that 15(65.22%) isolates of the *P. aeruginosa* out of 23 isolates have *pvd* gene, while 8 isolates did not have the *pvd* gene at a rate of (34.78 %). Additionally, the results partially agreement with a research (25) which reported that 38 (50%) of the 76 isolates had the *pvd* gene.

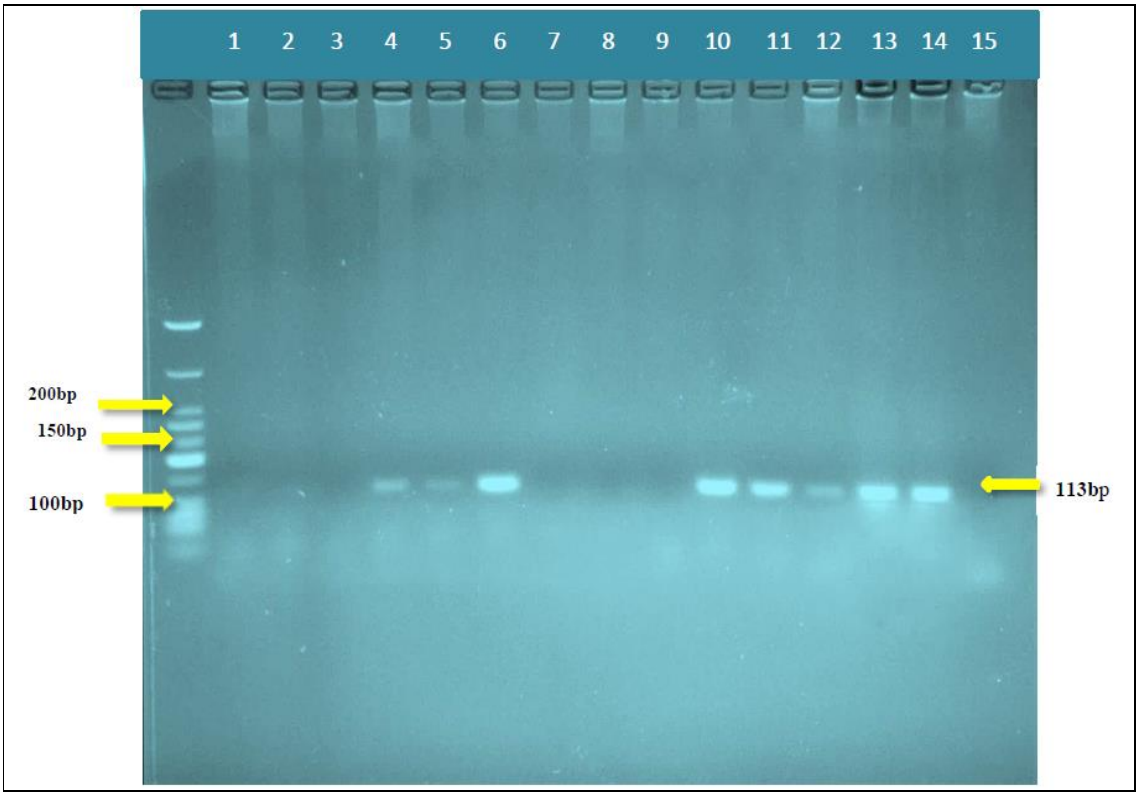

Figure (4): Gel electrophoresis of amplified PCR product of *pvd* Gene (113bp) of *P. aeruginosa* bacterial isolates at (90v. for 70min.) stained with red safe stain and were fractionated on 1.5% agarose gel electrophoresis. M: 25 bp ladder marker.

This study's conventional PCR detection of the *pch* gene in *P. aeruginosa* revealed that only 15 (50%) of the 30 isolates had this gene; the amplified PCR product for *pch* was shown on a gel electrophoresis in figure(5). These results contradict with a study which found that all isolates of *P. aeruginosa* had the *pch* gene (100%)(26). The other gene in *P. aeruginosa* studied in this work was *fur*, which was detected in 27 (90%) of the 30 isolates of *P. aeruginosa*. Figure (6) showed the amplified PCR product's gel electrophoresis.

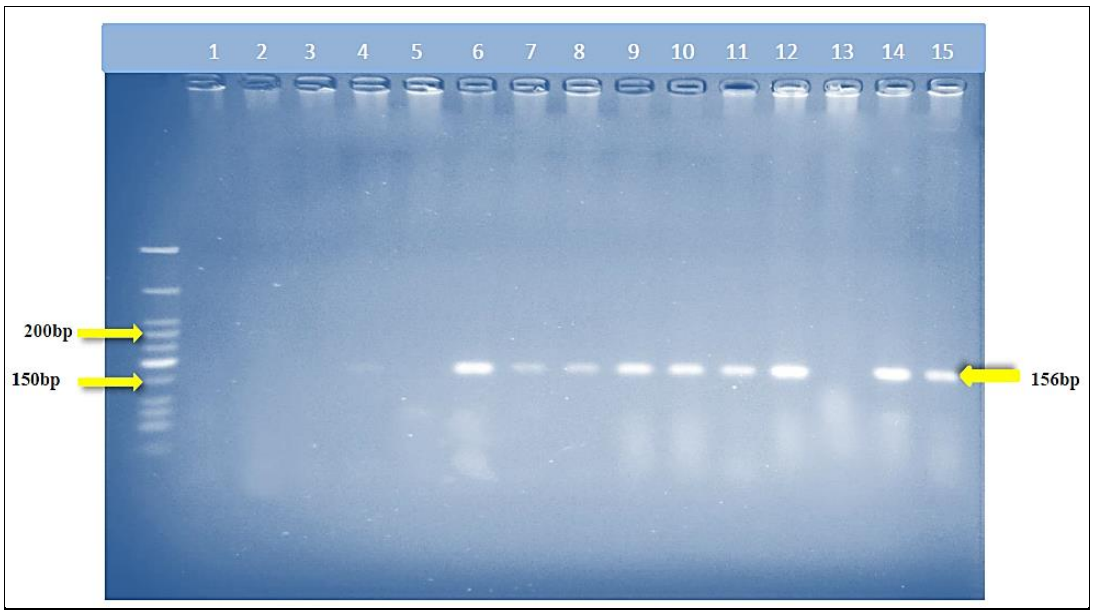

Figure (5): Gel electrophoresis of amplified PCR product of *pch* Gene (156bp) of *P.aeruginosa* bacterial isolates at(90v.for 70min.) stained with red safe stain and werefractionated on 1.5% agarose gel electrophoresis.M: 25 bp ladder marker.

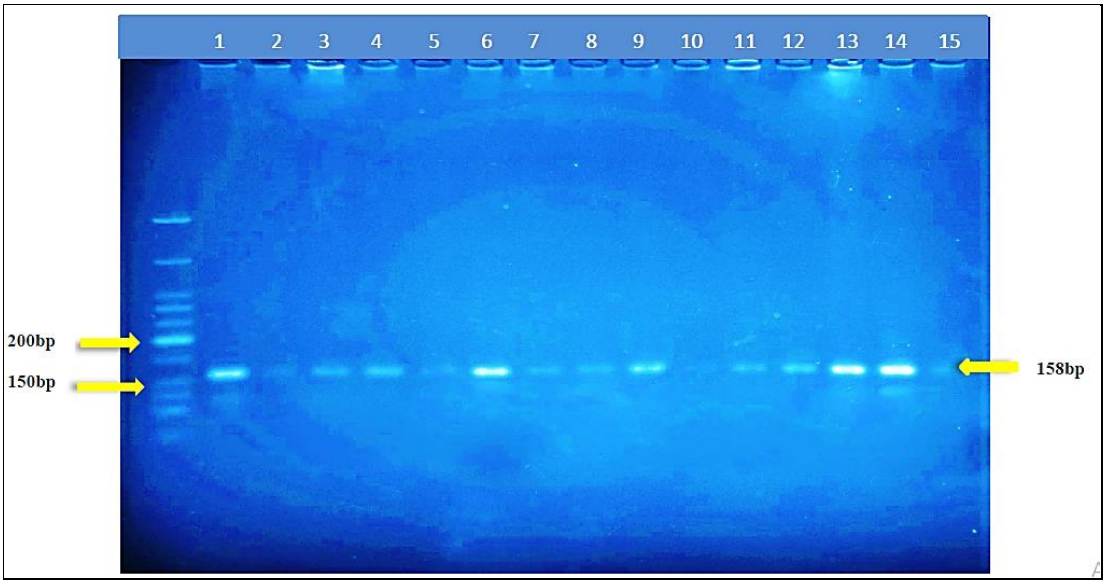

Figure (6): Gel electrophoresis of amplified PCR product of *fur* Gene (158bp) of *P. aeruginosa* bacterial isolates at (90v.for 70min.) stained with red safe stain and were fractionated on 1.5% agarose gel electrophoresis. M: 25 bp ladder marker.

Our results demonstrated that the relative frequencies of virulence genes were greater in a few distinct clinical samples. Some virulence determinants, which in turn affect the virulence of *P. aeruginosa* clinical isolates, have been postulated to be influenced by the duration of illness and the site of infection.As demonstrated by a study which revealed that 100% of *P.aeruginosa* isolates from wound samples were positive for *exoA* and *exoS*, some anatomical areas, for instance, increase the production of

exoA and *exoS* (27), while in a research in Poland (28) the 62 clinical isolates of *P.aeruginosa* had a *toxA* prevalence of 88.7%, while *exoS* prevalence of (75%). The presence of virulence genes was showed in Figure (7).

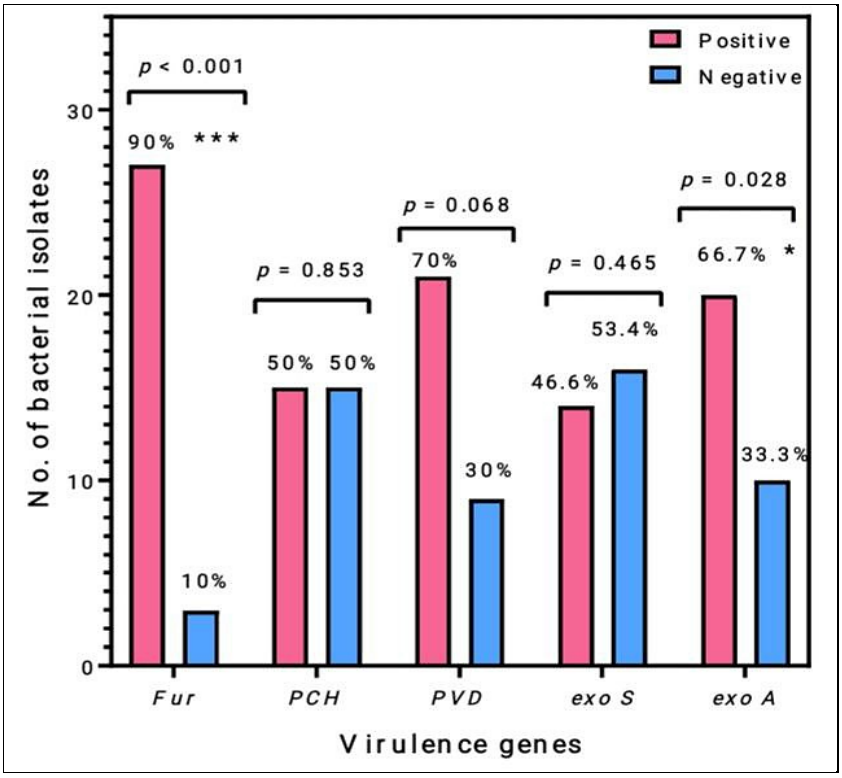

Figure (7): Presence of virulence genes detected with conventional PCR among clinical isolates of *Pseudomonas aeruginosa*. *p*: the probability of the Person Chi-squared test between categories variables. Asterisk (*) indicates statistically significant ($p < 0.001$ and $p < 0.05$).

Conclusion

According to the current study, *P. aeruginosa* was isolated at a higher rate 20 (66.6%) from burn patients compared to wound patients 10 (33.3%). The identification of virulence genes revealed that *P. aeruginosa* had different relative frequencies of virulence genes, 21(70%) of isolates were have *exo A*, 14(46.6%) out of 30 isolates had *exoS*, 21(70%) out of 30 isolates had *pvd*, 15(50%) had *pch* and 27(90%) out of 30 isolates had *fur* gene.

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