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RESEARCH ARTICLE

Comparative Study of Molecular Markers of *P.aeruginosa* Isolated From Clinical and Environmental

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Abstract

The present study includes (380) samples collected from clinical and environmental sources (clinical) ones included patients suffering from various infection and environmental samples . All samples were inoculated on selective media to detect *Pseudomonas aeruginosa* isolates .

Out of the 380 samples, only 116(30.5%) isolates belonged to *P. aeruginosa*, identified by diagnostic tests including cultural characters, biochemical tests and confirmed by PCR technique .

The distribution of these isolates were 61(62.0%) burn, 9 (9.2%), urine, 3(3.1), blood, 5(5.1%), sputum , 18(18.3%) ear swab , and 1(1.0%) each pleural and C.S.F, while environmental samples were 5(27.8) water , 3(16.7) soil, and 10(55.5%) sewage .Regarding to molecular diagnosis , the outcome showed molecular detection revealed that 116 isolates of *P. aeruginosa* were *16SrRNA gene* (which a specific of *P. aeruginosa*) . Genotypic detection of pathogenicity markers genes of *P.aeruginosa* were carrying (***plc-H***, ***plc-N***) as 89 (96%) for clinical isolate and (13(72%),14(78%) for environmental isolate, so the proportion of possession of ***rhIAB*** was as the following 89(96%) ,

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which is responsible for more infection in cystic fibrosis patients as well as for nosocomial infections in immunocompromised hosts. This bacterium determines many kinds of infections , *P.aeruginosa* is responsible for high rate of morbidity and mortality (Delden&Iglewski,2008)

P aeruginosa that normally lives in most environmenents. It has minimal nutrition requirement with being able to use several organic compounds for growth . This metabolite versatility contributes to abroad ecological adaptability , distribution, and reflect a genome of large size and complexity compared with that of many other bacterial species (Stover *et al*.,2000) .

The first step in *P.aeruginosa* infections is the colonization of the alerted epithelium, adherence of *P.aeruginosa* to the epithelium is mediated by pili and flagella. Evidence a implicates LPS as an adhesive factor. *P.aeruginosa* produced viscous exopolysaccharaied (alginate) in upper airway of patients with cystic fibrosis. After colonization *P.aeruginosa* produce several extracellular virulence factors alkaline protease,elastase, protease IV, rhamnolipid and two Phospholipase -C (haemolytic Phospholipase C) (PLC-H), and non-haemolytic Phospholipase C(PLC-N), neuraminidase and exotoxins responsible for extensive tissue damage, blood stream invasion and dissemination. Many of these extracellular virulence factors are controlled by cell to cell signaling system (Cotar *et al*.,2013) . The Interaction between virulence factors and the host immune response determinate the severity and the type of infections. In addition to the problems of high incidence and infection severity, the resistance of *P.aeruginosa* to conventional antimicrobial treatment has increased over the past decade (Kipnis *et al*.,2006) .

Materials and Methods :.

Samples Collection :.

A total of 380 samples were collected during the period from February to August 2013. The samples were divided into two groups as following:

A. Clinical Samples:.

Two hundred and sixty clinical samples of sputum, ear, urine and burn wounds were collected from patients visited/or admitted to three hospitals and laboratories in the Holly Najaf city included: Al-Sadder Medical City, Al-Hakeem General Hospital, Al-Zahra 'a Hospital for Childbirth and Children. Patients were tabulated according to their age, gender, hospitalization, antibiotic receiving and immunity state. Hence, the patients were considered as immunosuppressed if they have certain underlying diseases such as malignancy, cirrhosis, renal failure, and diabetes mellitus .

B. Environmental Samples:.

A total one hundred and twenty environmental samples were screened randomly from sewage, soil and water in Al- Najaf city.

1:.Isolation of *P.aeruginosa* :.

Bacterial identification by :.

Cultural Characteristics :.

All samples were cultured on MacConkey agar, blood agar, Pseudomonas Isolation Agar and nutrient agar media using standard loop method, The media were incubated at 37 ˚C for 24-48 hrs. Morphological colonies characteristics were recorded on the media that are used . The pure colonies were prepared for biochemical tests to differentiate *P. aeruginosa* from other bacteria .

Biochemical test :.

The finding of biochemical tests of *P. aeruginosa* showed that all isolates have the ability to produce Oxidase ,Catalase and grow at 42 °C, also, most of the isolates have the ability to reduce nitrates, hemolysis(β-hemolytic), and non-able to produce H**2**S. The results were according to standard references by compared with reverential reported by references .

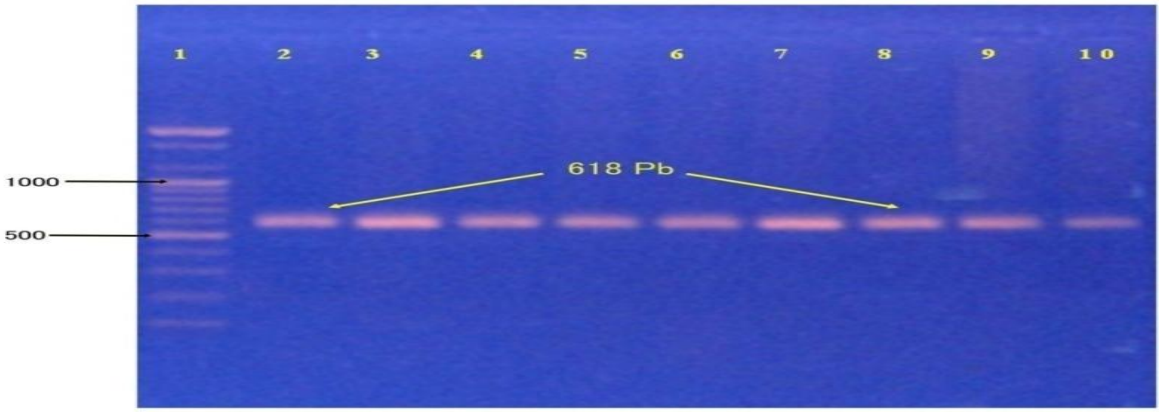
Identification of *P.aeruginosa* by PCR amplification of *16SrRNA*:.

Polymerase chain reaction technique of the *P. aeruginosa* clinical isolates revealed one fragment with 618 bp that represented the 16S RNA gene . The results show that all isolates of *P. aeruginosa* , carrying *16S r RNA* gene that is characteristic of *P. aeruginosa* in both clinical and environmental samples as shown in figure(1).

Results:.

Pathogenicity markers detection :.

Polymerase chain reaction technique of the *P. aeruginosa* clinical and environmental isolates revealed one fragment with 307 bp that represented the *plc-H* gene . The results showed that isolates of *P. aeruginosa* , carrying *plc-H RNA* are listed as clinical isolate show 89(96%) carrying *plc-H* . While environmental isolate showed 13(72%) positive for this gene .Clinical isolate distribution according is to source as burn 61(100%) , Ear 18(100%) , Urine 6(67%) , Sputum 4(80%) as shown as figure (2) .



Figure(1):PCR amplified products of *16S RNA* gene of the *P. aeruginosa* using the designed primers with expected size 618bp. Lane**s** (1), DNA marker (100bp ladder) , Lanes (2 ,3,4,5,6,7,8,9) positive results of *16Sr RNA* gene for *P. aeruginosa* isolates .

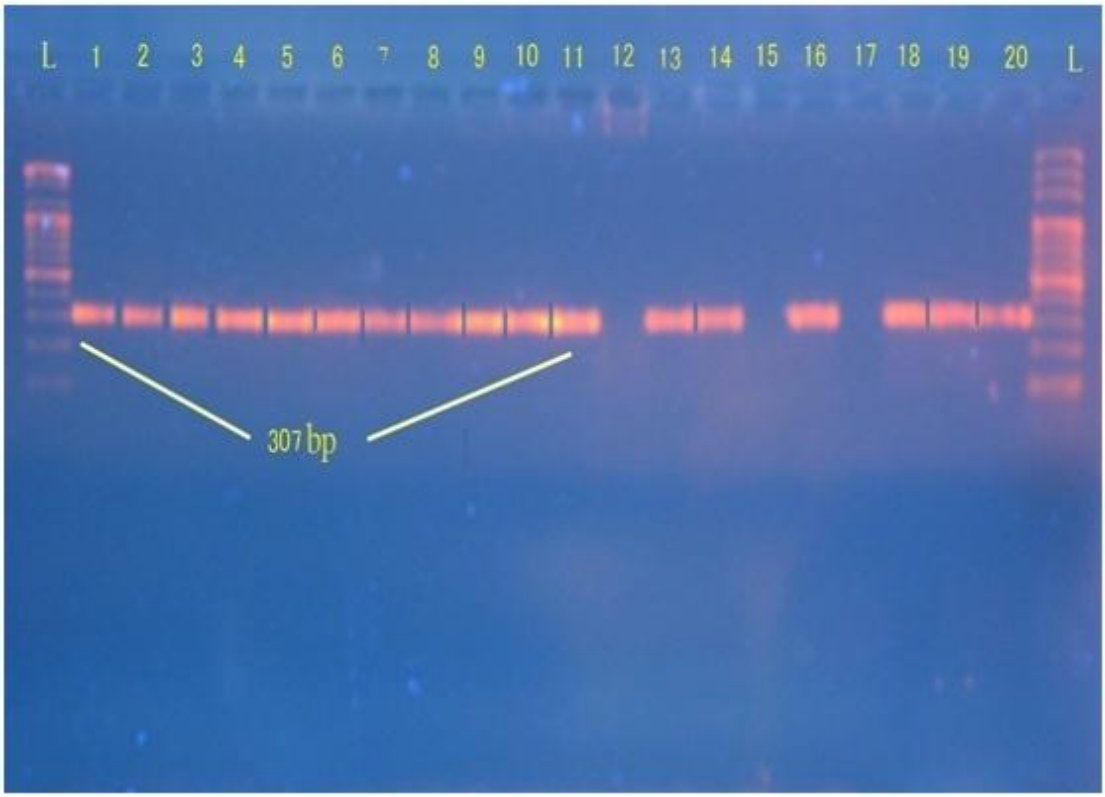


Figure (2): Ethidium bromide-stained 1.5% agarose gel showing the amplified product of(*plc-H*) .
Lanes (L): DNA marker (100bp ladder) . Lanes (1-4): burn samples ,Lanes (5-8): Ear samples , Lanes (9-12) Urine , Lanes (13-16) Sputum and Lanes (17-20) Environmental samples .

Regard to *(plc-N)* showed that all *P.aeruginosa* possesses (plc-N) except that only 4 isolates from environmental samples which do not possess this gene in addition 4 isolates from Urine samples donot posses this gene . The results as listed from 93 positive clinical isolate 89 (96%) posses this gene as burne 61(100%), Ear 18(100%). Urine 5(56 %) , Sputum 5(100%) while from 18 positive environmental samples only 14(78%) posses this gene as shown in figure (3) .

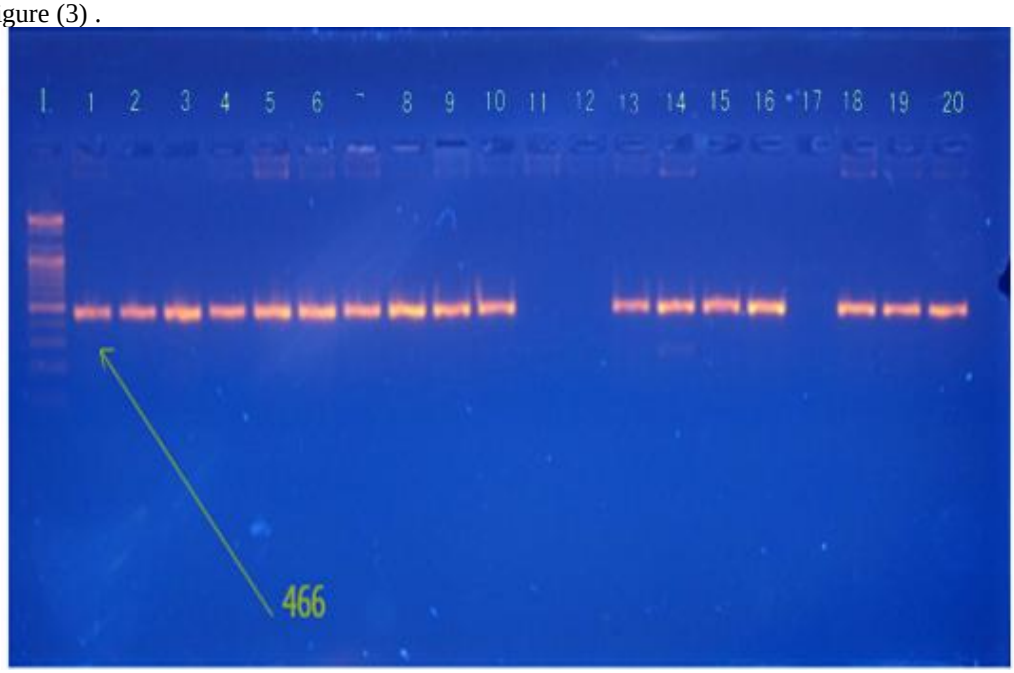


Figure (3): Ethidium bromide-stained 1.5% agarose gel showing the amplified product of *plc-N* gene.
L: DNA marker (100bp ladder) . Lanes (1-4): burn samples ,Lanes (5-8): Ear samples , Lanes (9-12) Urine , Lanes (13-16) Sputum and Lanes (17-20) Environmental samples .

The finding of PCR analysis showed that *P.aeruginosa* isolate possesses *rhIAB* gene, encoding the rhamnolipid distributed according to type and source of isolate as the follow from 93 positive isolate of clinical samples shows 89(96%) positive for *rhIAB* as follows burn 61(100%) , Ear 18(100%) ,Urine 5(56%) and sputum 5(100%) ,While from 18 positive isolate of environmental samples shows 13(72%) possesses *rhIAB* as shown in figure (4) .

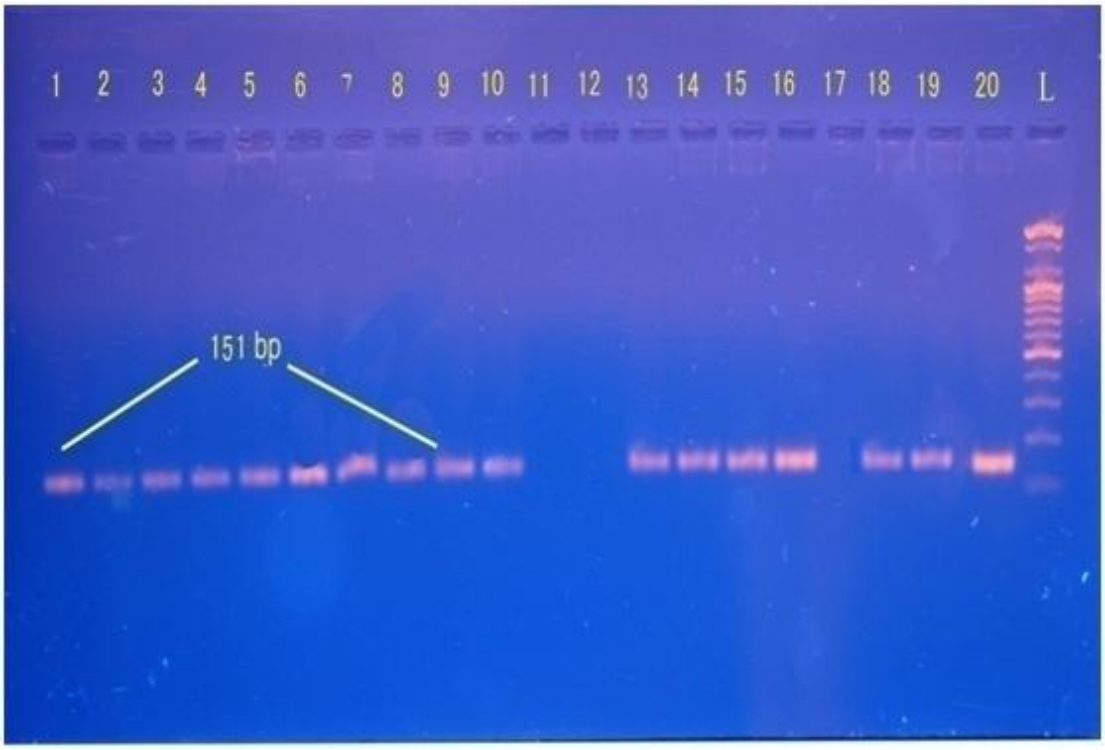


Figure (4): Ethidium bromide-stained 1.5% agarose gel showing the amplified product of *rhIAB* gene.
L: DNA marker (100bp ladder) . Lanes (1-4): burn samples ,Lanes (5-8): Ear samples , Lanes (9-12) Urine , Lanes (13-16) Sputum and Lanes (17-20) Environmental samples .

Table 1: Distribution of genotypic virulence determinants of *P.aeruginosa* isolates :.

Gene	Clinical isolate (no %+ve)	Environmental isolate (no%+ve)
Plc-H	89(96%)	13(72%)
Plc-N	89(96%)	14(78%)
rhIAB	89(96%)	13(72%)
16sRNA	93(100%)	18(100%)

The prevalence of pathogenicity markers genes were high rate of ***plc-H, plc-N*** and ***rhIAB*** in *P.aeruginosa* isolates 89(96%) while in environmental isolates showed 13(72%) for ***plc-H, plc-N*** and 14(78%) possesses of ***rhIAB*** .

Discussions

Identification of bacteria :.

In this study, out of 116 cultures positive *P.aeruginosa* isolate from 98 positive for clinical isolate show 85 (86.7) which were positive , while only 18 positive for environmental isolate show 12 (66.6%) were positive by cultural characters .

The Polymerase chain reaction technique of the *P. aeruginosa* clinical and environmental isolates revealed one fragment with 618 bp that represented the 16S RNA gene as shown in figure (1) . The results show that all of both isolates of *P. aeruginosa* (clinical and environmental) showed the results 98 (100%) as sensitivity 100% for clinical isolates in addition 18(100%) with sensitivity 100% for environmental isolates. This results similar to the study by (Prakash *et al*., 2005). In India, Prakash and others used the nested PCR with a different DNA extraction method and it was again better (53/57 positive 73.0%) than other method (Prakash *et al*., 2005). In another study, in Pakistan, 55 isolates of *P.aeruginosa* were diagnosed by the PCR gave 54(98%) positivity, , showing significantly better results by PCR (Haque *et al*., 2001).

A large, well designed study in Indonesia by Hatta and Smith reported a sensitivity of 94.5% by PCR from clinical and environmental samples (Hatta *et al*., 2007). It is, therefore, concluded that the PCR method was much superior to others tests yielding very high sensitivity and specificity. Although the PCR method requires extensive infrastructure and specialized skilled personnel, and cannot be made available everywhere, especially in developing countries, it can be made available to the reference centers for utilizations by other healthcare facilities following referral system. In fact, due to the rapid and definitive diagnosis, hospital admission of the patient can be avoided, reducing patients suffering, save working days and unnecessary expenditure on unrelated and misdirected treatment which may be many times more than the cost of PCR (Hatta *et al*., 2007).

Detection of pathogenicity Markers :.

Phospholipase C-Haemolytic (Plc-H) is a heat-labile haemolysin of sheep and human erythrocytes which has activity specific for the substrates sphingomyelin and phosphatidylcholine.

Although it is the second most extensively studied other than Plc-H, little is really known about the role of Phospholipase C-Non-Haemolytic (Plc-N) in pathogenesis (Vasil, 2006,). Plc-N is slightly smaller than Plc-H at 73KDa, shares about 40% amino acid homology with PLC-H and does not cause haemolysis of sheep or human erythrocytes (Ostroff *et al*, 2000).

The results of PCR analysis showed that not all *P. aeruginosa* isolates possess *plcN* gene, whereas *plcH* gene is present in all isolate except 4 clinical samples in addition to five environmental samples (Figures 2 and 3) while the results of PCR analysis showed that almost *P. aeruginosa* possess *rhlAB* gene, encoding the rhamnolipid except urine samples and some isolate of environmental (Figure 4) .

There are three other soluble proteins involved in *P. aeruginosa* invasion are represented by a rhamnolipid, two phospholipases C, haemolytic phospholipase C (*PLC-H*), and non-haemolytic phospholipase C (*PLC-N*). These factors may act synergistically to break down phospholipids (e.g., phosphatidylcholine and sphingomyelin), and contribute to invasion by means of their cytotoxic effects on neutrophils, lymphocytes and other eucaryotic cells(Finnan *et al*.,2004).

Rhamnolipid, a rhamnose-containing glycolipid biosurfactant, has a detergent-like structure that inhibits the mucociliary transport and ciliary function of human respiratory epithelium (Lanotte *et al*.,2004). Concerning to phospholipases C,. These two enzymes could act sequentially and synergistically, *PLC-H* would promote degradation of the erythrocyte membrane where as, *PLC-N* could then hydrolyze phospatidylserine present in the inner leaflet. The products of phospholipid hydrolysis would be digested by alkaline phosphatase, pyrophosphate being released. The experimental studies demonstrated that purified *PLC-H* determines vascular permeability, organ injuries, and death when injected in high doses into mice, as proving that *PLC-H* is an important virulence factor.

And through the results that appear in the figures above ,we find that our study is identical with a lot views , especially (Finnan *et al*.,2004;Gupta *et al*.,2006;Sadikot *et al*.,2008) in addition (Wilderman et al.,2001; Nathan and Yu ,2004).

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