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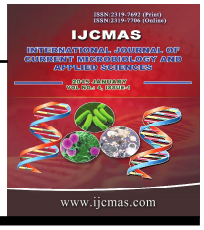

Original Research Article

Evaluation of Genetic Study and Bacterial Culture for Diagnosis of Pseudomonal Eye Infections

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A B S T R A C T

Human eyes acquire a microbial flora at birth and some of the commensal flora may become resident in the conjunctiva and eyelids with a potential to become pathogenic. Moreover, different types of bacteria invade the ocular tissues and causing infection. *Pseudomonas aeruginosa* is one of pathogenic agents that invade eye tissues and cause infection. The present study was designed as cross-sectional study to evaluate some virulence factor and bacterial culture for diagnosis of *P.aeuroginosa* isolated from eye infections from patients in Baghdad city. A total of 100 specimens(64 males and 36 females)there were 20(20%) positive isolates for *Pseudomonas aeruginosa*, which obtained from patients with different ages admitted to four hospitals in Baghdad city during the period from 18 January 2014 to15 May 2014. Results of genetic study were showed that for both Tox A gene and PLC gene which is responsible for disrupt host cell structures, and adhesions that allow for binding to epithelial cell receptors, the positive of *Pseudomonal* isolates were 18 (90%) and two isolates were negative 2(10%) withhighly significant differences (P<0.01). The study indicate that detection of virulence factors such as ToxA and phospholipase C gene by PCR was sensitive enough for the diagnosis of eye infections caused by *P.aeuroginosa*.

Keywords

Genetic Study,
Bacterial culture,
Eye infections,
P. aeuroginosa

Introduction

Infections of the eyes are commonly associated with microbial contamination of contact lenses. Whereas eyes can get infections from bacteria, fungi, viruses and other pathogenic agents. The most severe form of these infections is bacterial keratitis that if not treated properly, can lead to scarring and blindness (Willcox, 2007). Also the main microbial keratitis is *Pseudomonas aeruginosa*, which responsible for serious eye infections such as asymptomatic eye infections (Ashaye and Aimola, 2008). *Pseudomonas aeruginosa* is gram negative, obligate an aerobic and non sporulation, and is ubiquities organisms widely distributed in soil, water and living hosts and motile through polar flagellum (A kanji *et al*., 2011). *P. aeruginosa* commonly cause nosocomial outbreaks, especially in

immune suppressed and longtime hospitalized of intensive care unit patients (Brusselaers *et al*., 2013).

The pathogenesis of *P. aeruginosa* infections is multi factorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium. Pili, lipopolysaccharide (LPS), flagella, elastase, alkaline protease, siderophores, siderophore uptake systems and extracellular protein toxins (exoenzyme S and exotoxin A) are examples of other virulence factors (Todar *et al*., 2009). *P. aeruginosa* produces several phospholipases, hemolytic Plc-H and non-hemolytic Plc-N, Plc-B (Barker *et al*., 2004). Investigations of the *P. aeruginosa* have been hampered by the inadequate discriminatory capacity of classical phenotypic methods such as serotyping, phage typing, pyocin typing and biotyping in classical microbiology phenotypical, serotypical methods and PCR technique are used to diagnosis and differentiate between bacterial species (Kiska and Gilligan, 2003; Bergogne-Berezin, 2004).

The ToxA gene which is responsible for inhibition of protein synthesis and causes tissue damage of cornea. Song *et al*.,(2000) applied that the PCR technique was useful for detecting *P. aeruginosa* from eye infections isolates by targeting its Exotoxin A gene. The study aimed to evaluate the genetic methods by PCR detection of toxin A and phospholipase C in addition to bacterial culture for diagnosis of Pseudomonal eye infections isolates through some patients in Baghdad city.

Materials and Methods

Patients: One hundred samples were collected from patients including (64 males and 36 females) with age ranged from less than 10 to 70 years, who suffered from eye infections diagnosed by ophthalmologists. Patients were presenting to four hospitals in Baghdad city during the period from 18 January 2014 to15 May 2014.

Specimen collection

Eye specimens from the external ocular surface were collected using cotton swabs and corneal scrapes. These specimens were directly inoculated onto the plates of transport medium, then incubated overnight at 37˚C (Mims *et al*., 2008).The history for each case was recorded in a questionnaire.

Identification of *P. aeruginosa*

Morphological Examination:

Colonies that grow on the selective media were further identified by studying their morphological characteristics beginning by Gram stain and appearance under light microscope (Gram reaction, shape, arrangements) (Collee *et al.*, 1996).

Culturing of eye specimens: Collected specimens were inoculated into three culture media, which were Blood agar, MacConkey agar**,** and Cetrimide agar (Morello *et al*., 2006).

Biochemical Tests

Different biochemical tests were achieved for the suspected colonies, which include oxidase test, catalase test, indole production test, methyl red test, voges-proskaur test, simmon's citrate utilization test. The identification of the isolates further was carried out by Growth at 42°C,Growth at 4°C, only *P.aeuroginosa* colonies which have this ability.

Biochemical tests were performed to support results using API 20NE, which is accurate technique for Confirm identification of *P.aeuroginosa* isolates (O'Hara, 2003). Also

Vitek2 was used for confirmation of identification of suspected isolates of *P. aeruginosa* by used card 2GN (biomeriux company, France)**.**

Molecular study

Detection of ToxA and *PLcH* genes by PCR Assay:

Genomic DNA was extracted from *P. aeruginosa* strains using ZR Fungal/Bacterial DNA MiniPrep™ kit Following manufacture instructions. The sequences of primer sets were used in PCR to amplify species were shown in Table (1).

Amplification of *ToxA and PlcH* genes of *pseudomonas aeruginosa*:

Briefly, the (***ToxA andPlcH***) genes were studied according to protocol of Rosario *et al*.,(2012).This was done by using specific primers. The PCR reaction mixture contained 12.5 µl of green master mix (containing bacterially derived *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 5 µl of template DNA, 1.5 µl of each forward and reverse primers, then the volume completed to 25 µlby free - nuclease water.

All tubes then shook well then PCR tubes were transferred to the thermalcycler (eppendroff, Germany) to start the amplification reaction according to specific program for each gene initial denaturation cycle at 94˚**C** for 2 min, followed by 35 cycles to denaturation at 94˚**C** for 1 min, annealing temperature 54˚**C** for 1 min, extension step72˚**C** for 1 min and final cycle of 72˚Cfor 2 min. Amplified products were electrophoresed on 1.5% agarose for 1hr at 75 V/cm.

Statistical analysis

Statistical programs performed by SPSS (V-15) and figures by Excel 2007 in (P5) computer. Descriptive statistics were included tables creation (number, percentage, mean and standard error) and Graphical presentation such as figures. While inferential statistics include Chi-square, t-test and Z-test (McCall, 1980).

Result and Discussion

Isolation and Identification

A total of one hundred samples were obtained from eye infections patients presenting to four hospitals in Baghdad city. The main characterization phenotypic tests were used for isolation and identification of isolated bacteria such as cellular characteristics, cultural morphology and biochemical tests which are confirmed by using of Mini API 20NE method andVitek2 system. These tests were selected as conventional tests to identify the isolated bacteria (Collee *et al*., 1996).

Phenotypic characteristics

Colonies morphology and cells characteristics

Twenty isolates (20%) of *Pseudomonas aeruginosa* were obtained, that showed G-ve straight rods, motile and colonies on MacConkey agar appeared as small round convex colonies with pale colour (lactose non-fermenting), either smooth or rough colonies with regular edges. Also they produced grape-like or rotten-potato odor, and most isolates exhibited β-hemolysis on blood agar after 24 hour of incubation (Collee *et al*., 1996).

Isolates of *P.aeuroginosa* were showed ability to produce pyocyanin pigment on cetrimide agar which enhance its virulence (O'Mally *et al*., 2004), Pigments were genetically encoded by two operons for the production of metabolites such as pyocyanin, andpyoverdin (Kennth, 2011).(Figure 1)

Biochemical tests

All *pseudomonas* isolates were positive for oxidase, catalase, Simmon's citrate utilization, and negative result for indole and MR-VP tests in this study. It does not ferment carbohydrates, but many strains oxidize glucose, which confirmative by Mini API 20NE system and Vitek2 system (Brooks *et al*., 2010).

Frequency of *Pseudomonas* isolates

In this study the most commonly isolated bacterium was *P. aeruginosa* which forms 20(20%) from total cases while no growth 80(80%) as listed in table(2) which appear highly significant differences (P<0.01) among cultured results. These findings agree with Tesfaye*et al*.,(2013) who reported that the predominant isolate was *P. aeruginosa* (20.9%) and nearly to Mohammed,(2014) who reported *P.aeruginosa* isolated from eye was 18(18.36%) from total isolate(23) .

Blood agar haemolysis in relation to culture result

The predominant in this study Beta haemolysis isolates were 16(80%), while Alpha 4(20%) with significant differences (P<0.05).These result nearly to Jácome *et al*.,(2012)who reported the analysis of virulence factors revealed that out of the 61 *P. aeruginosa* isolates studied, while 57(93.4%) were Beta Hemolysin. Also Flayyih *et al*., (2013) showed that all the isolates100 (100%) had the ability to produce Beta Hemolysin these result disagree with current study**.**

Genotypic detection of isolates

Confirmative identification of *Pseudomonas* by amplification of ToxA and *PLcH* genes through PCR Assay

The ToxA gene which is responsible for inhibition of protein synthesis and causes tissue damage of cornea. The result presented in (Figure 2) showed that all *Pseudomonal* isolates except two isolate (L6 andL15) which was showed positive results (clear band) on 1.5% agarose gel as a result of PCR reactions, the size of the 454bp of the DNA ladder (100-1500bp)

Table (3) showed that positive Tox A gene of *Pseudomonal* isolate was 18 (90%), two isolate was negative 2(10%) with highly significant differences (P<0.01).These result agree with Song *et al*.,(2000) who applied that using PCR technique for detection *P. aeruginosa* from ocular isolates by targeting its Exotoxin A gene which showed only the strains of *P. aeruginosa* gave a distinct 5(100%) positive result and well defined 367-bp DNA band when analysed on an agarose gel electrophoresis. While Ghadaksaz *et al*., (2015) were reported the frequency distribution of the toxA genes in the clinical isolates was (84.6%).These result nearly with current study. In order that Sabharwal *et al*.,(2014) were reported that 12(100%) prevalence of ToxA gene was found in all analyzed strains, which was approach with current study results.

The PLC gene which is responsible for disrupt host cell structures, and adhesions that allow for binding to epithelial cell receptors. The results presented in (Figure 3) showed that all *Pseudomonal* isolates except

two isolate (L2 and L13) which was showed positive results (clear band) on 1.5% agarose gel as a result of PCR reactions, the size of the 447bp of the DNA ladder (100-1500bp).

In the study positive phospholipase C gene of *Pseudomonal* isolates were 18 (90%), two isolateswere negative 2(10%) and highly significant differences (P<0.01).Zahreddine *et al*., (2012)were showed that the virulence encoding gene PLC present in 46(100%) of isolates, these results approach with present study. So that Mohammad, (2013) who reported that (87.5%) from total 72Transport eyeswabs of isolates were phospholipase C, these result nearly to present study. While Sabharwal *et al*., (2014) were reported the prevalence of phospholipase C gene that 9(75%) found in all analyzed strains, so these findings differ from present study. Ugargol*et al*., (2014) showed that among 250 *P. aeruginosa* clinical isolates, 125(50%) produced Phospholipase C. These obtained results differ from current study.While that Fazeli and Momtaz,(2014) were indicated that the high presence of virulence factors in *P. aeruginosa*plcH isolates were (45.09%) and these findings not harmony with present study.

In conclusion the study indicate that according these demonstratedresults the detection of virulence factors such asToxA and phospholipase C gene by PCR was sensitive enough to be used for thediagnosis of eye infections caused by *P.aeuroginosa*.

Table.1 Oligonucleotide primers sequence used for PCR amplification of specific gene

Genes	Sequence (5' to 3')	Size (bp)	References
ToxA	**F**-TCAGGGCGCACGAGAGCAACGAGA	454	Rosario *et al*.,2012
	R-GACAGCCGCGCCGCCAGGTAGAGG		
plcH	**F**-CGACGAGGGCGACGGCTTCTATGA	447	Rosario *et al*.,2012
	R-CCGGGCAGGCTCTTGGGCTCGTA		

Table.2 Frequency of Pseudomonas isolates

GROWTH		N	%	Z-test (P-value)
Culture Result	Pseudomonas	20	20%	P=0.00 HS (P<0.01)
	No growth	80	80%	
	Total	100	100%	
Blood agar Haemolysis	Alpha	4	20%	P=0.012 S (P<0.05)
	Beta	16	80%	
	Total	20	100%	

Figure.1 Pseudomonas isolate growth on Cetrimide agar

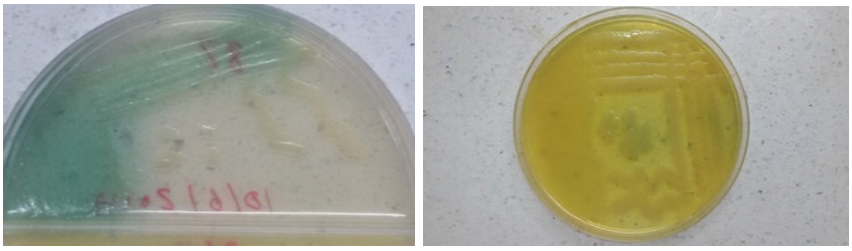


Table.3 Distribution of virulence factors among pseudomonal isolates

PCR		N	%	Z-test (P-value)
Tox A gene (PCR)	Positive	18	90%	P=0.00 HS (P<0.01)
	Negative	2	10%	
	Total	20	100%	
Phospho lipase C (PCR)	Positive	18	90%	P=0.00 HS (P<0.01)
	Negative	2	10%	
	Total	20	100%	
Total PCR	Positive	16	80%	P=0.012 S (P<0.05)
	Negative	4	20%	
	Total	20	100%	

Figure.2 Agarose gel electrophoresis (1.5% agarose, 75 V/cm for 1 hour) of Tox A PCR products (454bp amplicon) in P.aeuroginosa isolates. Lane L (DNA ladder) 100-1500bp molecular marker, lanes 1,2,3,5, 7,8,9,10,11,12,13,14 isolates are positive results while lane6 and 15 show negative results

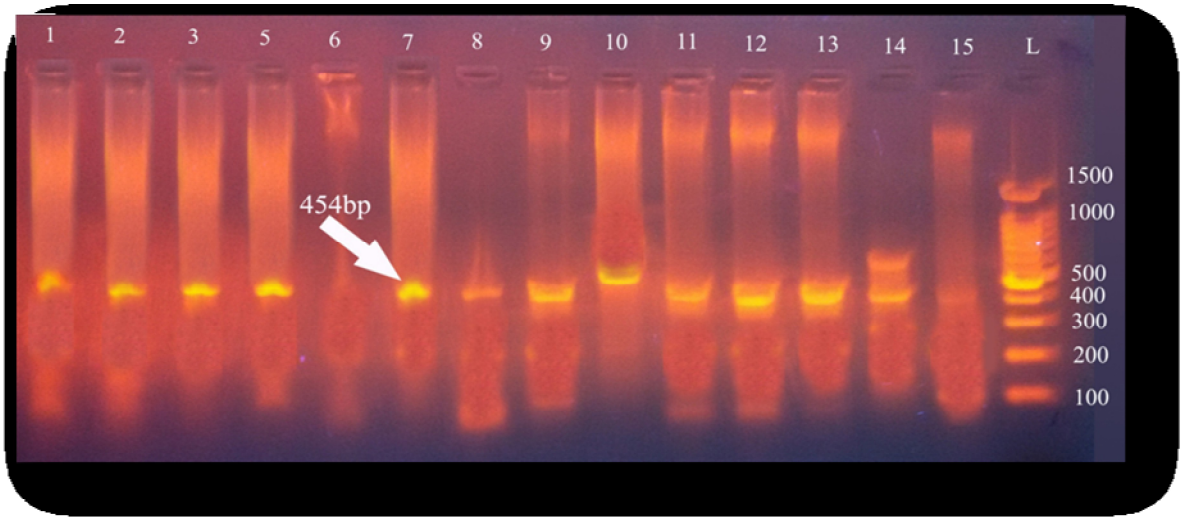
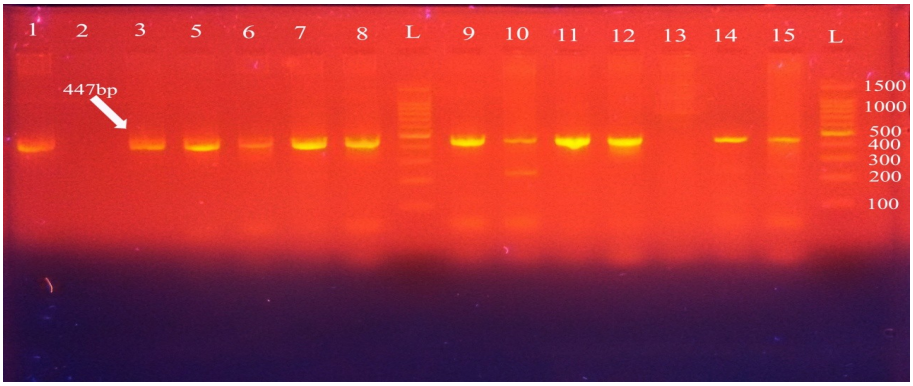


Figure.3 Agarose gel electrophoresis (1.5% agarose, 75 V/cm for 1 hour) of PLC PCR products (447bp amplicon) in P.aeuroginosa isolates. Lane L (DNA ladder) 100-1500bp molecular marker, lanes 1, 3, 5,6,7,8, 9,10,11,12, 14, 15 isolates are positive results while lane2 and 13 show negative results



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