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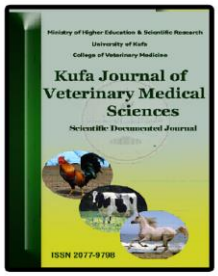

Molecular Detection of virulence factor genes in *pseudomonas aeruginosa* isolated from human and animals in Diwaniya province

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Abstract

Virulent factors are molecules that produced by pathogenic Pseudomonas aeruginosa that enable it to play a great role in causes of the disease , these virulence factors encoding by virulence genes located in the chromosome of Pseudomonas aeruginosa. However, this study was examined four virulence factors genes included (toxA) that encoding to exotoxin A , (exoS) that encoding to exoenzyme S , outer membrane protein (oprL), and outer membrane lipoprotein I (oprI) that encoding surface protein (lipoprotein) by use PCR technique based using specific primers were design in this study.(50) swab samples were collected from human clinical infection samples from Al-Diwanyia hospital, and (30) milk samples were taken from cattle milk that infected by mastitis from different fields in Al-Diwanyia province. The samples were cultured on selective media and then extracted DNA followed by PCR technique to detect the virulence genes. The results were shown a clear difference in isolation of P. areuginosa isolates from human and animal source. Where, in human the percentage of P. areuginosa in wound was (42.8%),in the burn was (70%) , and Otitis media was (68.7%), while, in cattle the percentage of P.aeruginosa in milk was (26.6%); Where percentage of ToxA genes was (100%) in the wound and cow milk; and it was (92.8%) and (81.8%) in burn and Otitis media respectively. while , exoA gene were (66%) , (57.1%), (45.4%) and (75%) while were oprL gene was (50%) ,(50%) , (54.5%) and (50%) , oprI were (66%) ,(42.8%) , (54.5%) and (50%) in Wound , Burn, Otitis media , Cattle milk isolates respectively **.**In conclusion,use of PCR technique which given high specific and accurate results for detection of virulence factors genes in P.aeruginosa isolates that responsible for cause in disease in human and animals.

Keyword :*Pseudomonas aeruginosa* **,** virulence factor genes**,** human and animals, PCR**.**

التشخيص الجزيئي لجينات عوامل الضراوة في الزوائف الزنجارية المعزولة من الانسان والحيوان في محافظة الديوانية

الخلاصة :

جزيئات عوامل الضراوة تنتج بواسطة الزوائف الزنجارية المرضية التي تلعب دورا كبيرا في سبب بعض الامراض ,هذه عوامل الضراوة تشفر بواسطة جينات الضراوة الموجودة في مورثات جرثومة الزوائف الزنجارية . ان هذه الدراسة اختبرت اربعة جينات تتضمن (تي او اكس اي) الذي يشفر لبروتين الاكسوتوكسين اي والمورثة (اي اكس او اس) الذي يشفر لبروتين (اكسوانزايم اس) , وجين بروتين الغلاف الخارجي ال (او بي ال) , وبروتين الغلاف الخارجي اي (او بي اي) بواسطة استخدام تفاعل السلسلة المتعدد طبقا الى استخدام بادئات خاصة صممت لهذه الدراسة .خمسين عزلة او مسحة جمعت من عزلات اصابات سريرية من مستشفى الديوانية وثلاثين عزلة حليب اخذت من ابقار مصابة بواسطة التهاب الضرع في حقول مختلفة في محافظة الديوانية . هذه العينات زرعت على اوساط زرعيه انتخابية ومن ثم استخلص المادة الوراثية (الدي ان اي) بواسطة تقنية تفاعل السلسلة المتعدد لتشخيص مورثات الضراوة . النتائج اظهرت اختلاف واضح في عزلات الزوائف الزنجارية من المصادر البشرية والمصادر الحيوانية حيث في البشر فان نسبة الزوائف الزنجارية في الجروح كانت (42.8%) وفي الحروق كانت (70%) اما الاذن الوسطى كانت (68.7%) بينما في الابقار كانت (26.6%). حيث كانت نسبة المورثة (تي اكس او اي) كانت (100%) في الجروح وحليب الابقار وكانت (92.8%) و (81.8%) في الجروح والاذن الوسطى على التوالي . بينما كانت المورثة (اي اكس او اي) كانت (66%), (57.1%) , (45.4%) و (75%) بينما جين (او بي ار ال) كانت (50%) ,(50%) ,(54.5%) و (50%) والمورثة (او بي ار اي) (66%) , (42.8%) ,(54.5%) و (50%) في عزلات الجروح والحروق والاذن الوسطى وحليب الابقار على التوالي . نستنتج ان تقنية تفاعل السلسلة المتعدد اعطى خصوصية عالية ونتائج دقيقة في تشخيص جينات عوامل الضراوة في جرثومة الزوائف الزنجارية المسؤولة عن احداث امراض في البشر والحيوانات

Introduction

Nosocomial infections are caused by several of organisms, e.g. bacteria, viruses, fungi, parasites, and other agents. Infections can be derived from exogenous or endogenous sources are transferred by either direct or indirect contact between patients, healthcare workers, contaminated objects, visitors, or even various environmental sources (1).

A second study conducted in 2002 estimated that when taking into account all types of bacterial infections, approximately 1.7million patients suffered from, which contributed to the deaths of 99,000 patients per year where considered *Pseudomonas aeruginosa* one most common (2).

P. aeruginosa is an Gram negative aerobic organism that is wide spread in nature. Many be reside in the water,plants, soil, and the predisposing environment, like hospital locations; opportunistic bacteria in the humans and animals (3).

P. aeruginosa is an opportunistic bacterium capable of infecting animals humans (4). the bacteria can be cause acute infections in all tissues. infection in Pulmonary tract infection by *P. aeruginosa* is the main disease (5). *P. aeruginosa* infections cases accrue inmost hospitals affect most patients in concentrated care units with burn wound infections or chronic disease (6) and (7).

Pseudomonas aeruginosa is a bacterium characterized by its high genetic plasticity and potential for adapting to various environments. The species are frequently isolated from soil and water or colonize several anatomical sites such as plants, insects, animals, and humans. The bacterium may be involved in food poisoning and has many virulence factors and it considered main cause for mastitis in dairy cattle(8) (9)(10)(11).

P. aeruginosa is a most common nosocomial in the human pathogen in immuno-compromised patients (13) like cancer and burns,(14)also this bacteria can effect the causing some

disease include pneumonia, endocarditis (15) and inflammation in the urinary tract (16) , there are some organs may be effect by this bacteria like central nervous system,eyes, ears, skin, wounds, and musculoskeletal system and causing cystic fibrosis, burns, and immunodeficiency(17)(18)(19)and(20)
.

P. aeruginosa considered have a large number of virulence factors suchas exoenzyme S ,exotoxin A, , elastase and sialidasean (21) addition to several of the other extra cellular products. Exotoxin Aencoded by the *toxA* gene which has the ability to inhibit protein biosynthesis just like diphtheria toxin(22)(23).

The aims of the present study were to investigate the percentage of some virulence genes in clinical cases of *P. aeruginosa* get from different disease and from human and animal including *exoS*, *toxA* and OPRL and OPTI by use PCR technique.

Materials and Methods:

Sample collection:

(50) swab samples were collected from human clinical infection samples including (Wound, burn, and otitis media infection)from Al-Diwanyia hospital. (30)milk samples from cattle infected by mastitis from different fields in Al-Diwanyia city. The samples placed in sterile transport media then transferred into microbiology Laboratory College of veterinary medicine and store in the refrigerator until bacterial isolation.

Bacterial isolation:

Pseudomonas aeruginosa was isolated from fecal samples by inoculation over Brain Heart Infusion media at (37°C) overnight in primary enrichment culture and then the bacterial growth was inoculated on chrome agar at 37°C overnight for selective isolation of pure culture *Pseudomonas aeruginosa* isolates.

Bacterial DNA extraction and PCR Method:

PCR technique was performed for virulence factors genes (exotoxin A (toxA), exoenzyme S (exoS), outer membrane protein (oprL) , and outer membrane lipoprotein I (oprI) gene in *Pseudomonas aeruginosa* based using specific primers were design in this study as following steps:-

1-DNA extraction: The bacterial isolates were subjected to bacterial nucleic acid extraction by using commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA**.** The extraction method was don depend on the manufacturing instructions by using gram positive bacteria DNA Protocol extraction method by using (20 mg/ml) lysozyme buffer.

2-Nanodrop: The extracted DNA was estimated by nanodrop device at 260/280nm, and then kept at deep freezer for used in PCR method.

3-Primers: The primers used in our study for diagnosis of virulence factors genes were designed in the study used NCBI Gene sequence information base and website primer 3 plus for design of primers; These primers were prepared by Bioneer company, in the Korea see below table(1):-

Table (1): primers and their sequence and GenBank codes:

Primer	Sequence (5'-3')		Amplicon	GenBank code
ToxA gene	F	CAGAACTGGACGGTGGAGC	535bp	AF227424.1
	R	CCTGTTCCTTGTCGGGGATG		
exoS gene	F	TCAGCAGAGTCCGTCTTTCG	680bp	AY029240.1
	R	CCGATACTCTGCTGACCTCG		
oprL gene	F	CGCGTCGAGCTGAAGAAGTA	860bp	AF177774.1
	R	CGGGATCAGCGAAGGTTCTT		
oprI gene	F	TGCAGCAACTCTACCCAAGG	312bp	M25761.1
	R	GGTTTCTTTGGAGTGGCTGC		

4- (PCR master mixture) preparation: The mix was treat by using (Accu-Power®PCR-PreMix-Kit) master mix reagent and it depended on company direction see table(2).

Table (2): company instructions of PCR master mix:-

Master mix	Volume
DNA template (10ng/µL)	5µL
Forward primer (10pmol)	1 µL
Reverse primer (10pmol)	1 µL
PCR water	12 µL
Total volume	20

ThePCR mixture revealed in the table above placed in AccuPower PCR -PreMix that contains all PCR components (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes keep at tube to transfer into vortex vibration for 3 minutes; transferred into thermocycler apparatus (MyGene, Bioneer. Korea).

5- PCR thermocycler conditions:

Table (3): PCR thermocycler conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	30 cycle
Annealing	57.2	30sec	
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

6- PCR product analysis: has examined by Electrophoresis apparatus in a 1% agarose substance by using buffer, then stained with ethidium bromide, and take alook under UV transilluminator.

Results :

Bacterial isolation results of *Pseudomonas aeruginosa* isolation results were shown in the following table(4):

Table(4): show number and percentage of isolate in wound, burn and otitis media

Sample type	Total samples	Positive isolate	Percent%
Wound	14	6	42.8%
Burn	20	14	70.0%
Otitis media	16	11	68.7%
Cattle Milk samples	30	8	26.6%

Chi-square statistical significant at (P < 0.05)

PCR was appeared specific assay that used in the detection of virulence factors genes(ToxA, exoS, OrpL, and OrpI) producing *Pseudomonas aeruginosa* isolates human infections patients and cattle milk samples. Where The PCR results were shown in following table(5).

Table (5) : Show number and percentage of the isolates for virulence gene from *P. aeruginosa* .

Virulence gene	Human clinical infection isolates			Cattle milk isolates (8)
	Wound (6)	Burn (14)	Otitis media (11)	
ToxA	6 (100%)	13 (92.8%)	9 (81.8%)	8 (100%)
exoA	4 (66%)	8 (57.1%)	5 (45.4%)	6 (75%)
oprL	3 (50%)	7 (50%)	6 (54.5%)	4 (50%)
oprI	4 (66%)	6 (42.8%)	6 (54.5%)	4 (50%)

Chi-square statistical significant at (P < 0.05)

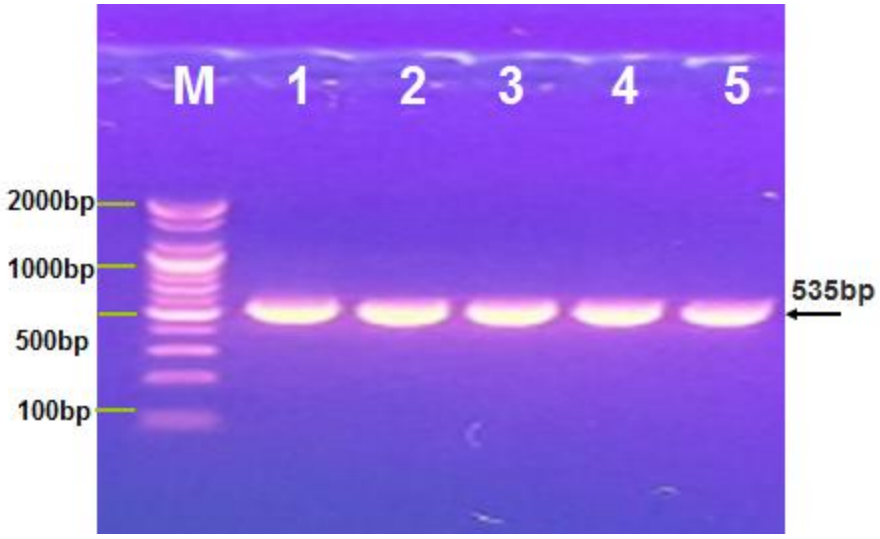

Figure (1): on Agarose in the electrophoresis of PCR assay reveal some positive results of exotoxinA toxA gene; DNA marker (2000-100bp)Lane ; (1-5) positive samples 535bp for exoSgene in *Pseudomonas aeruginosa* isolate.

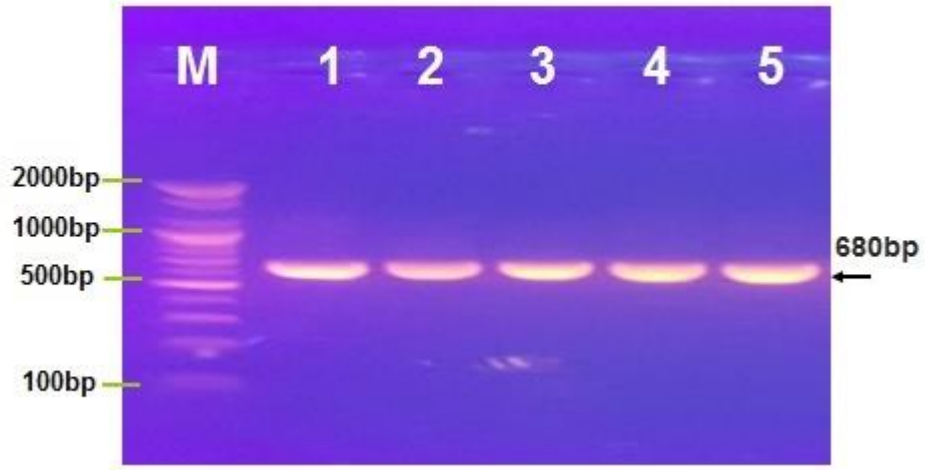

Figure (2): Agarose material on the electrophoresis of PCR assay see some positive results of exotoxin exoS gene; DNA marker (2000-100)bp, Lane (1-5) some positive isolates at 680bp for exoS gene in *Pseudomonas aeruginosa* isolates.

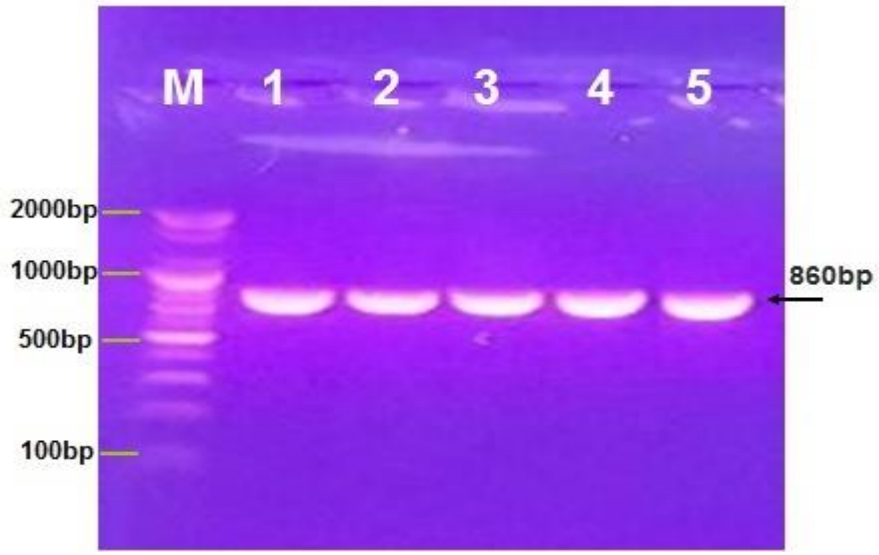

Figure (3): Agarose photo on electrophoresis of PCR assay; can see some positive results of Lipoprotein L gene; DNA marker (2000-100) bp, Where, Lane (M) DNA marker (2000-100)bp, Lane (1-5) some band positive isolates at 860bp for OrpL gene in *Pseudomonas aeruginosa* isolates.

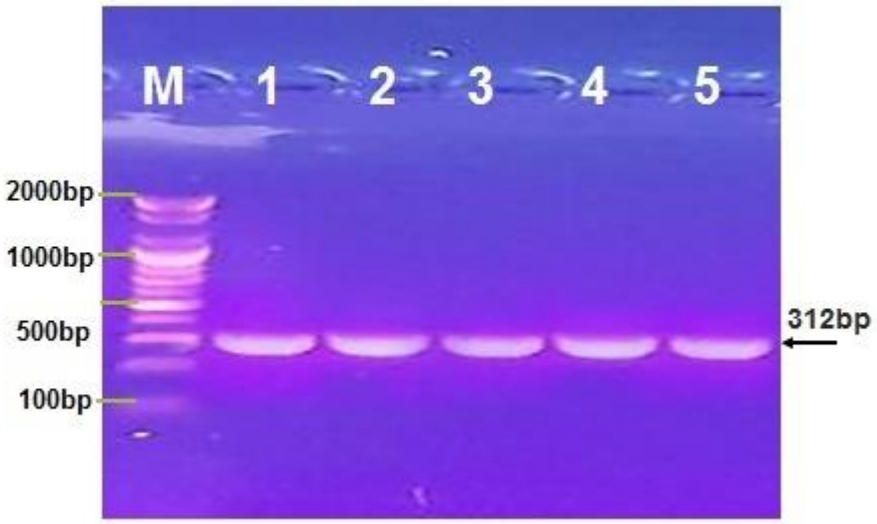

Figure (4): Agarose gel electrophoresis of PCR assay show the some positive results of Lipoprotein I gene in *P. aeruginosa*. DNA marker (2000-100) bp, Lane (1-5) some and positive isolates at 312bp for OrpI gene in *Pseudomonas aeruginosa*isolates.

Discussion :

Pseudomonas aeruginosa produces many of virulence factors (24) whose expression is arranged by different systems (25) A recent studies reveal *P. aeruginosa* is most frequent pathogen that formed many of virulence factors exampleToxA,exoA , oprL and oprI genes (26) (27) and (28).

In our studyIn human the percentage of *pseudomonas aruegenosa in the wound was* 42.8% , in the burn was 70%, and Otitis media was 68.7%, while, in cattle, the percentage of *pseudomonas aruegenosa in milk was* 26.6% .

Where percentage of ToxA genes was (100%) in the wound and cow milk and it was (92.8%) and (81.8%) in burn and Otitis media respectivily.

While , exoA gene were (66%) , (57.1%), (45.4%) and (75%) while were oprL gene was (50%) ,(50%) , (54.5%) and (50%) , oprI were (66%) , (42.8%) , (54.5%) and (50%) in Wound , Burn, Otitis media , Cattle milk isolates respectively **.**

(29) examined (26) clinical isolates of bacteria got from the patients that submitted and tested in Baghdad hospitals/Iraq (22) isolates has taken from different infection; (4) isolates from cystic fibrosis patients. The percentage of virulence genes, exoenzyme S (*exoS*), exotoxin A (*toxA*) were determined by PCR. The most common were *exoS* where detected in 21/26 (80.7%) as a percentage and distributed between (19) in different clinical source and (4) in CF samples. *toxA* were 19/26 (73%) where this study near close to our results.

While (30) have a rate of toxA gene higher percentage from our study where it was (100%) in all analyzed strains was found to be related to the presence.

The virulence of *P. aeruginosa* is many of extracellular enzymes and

another exotoxin ; these virulence factors are very important to the pathogenesis of tissue for *P.aeruginosa* infections (31).

The study (31)found *exoS* gene was (80.7%),and that near to our results and (32) agreement too where reported that(75.3%) of their isolates produce positive results for *exoS ;* while the results of (32) and (33) close to our study, when they recorded(58.6%), (65.0%) respectively ;the presence of *exoS* gene in isolates from cystic fibrosis patients, wound, urine, and blood was significantly higher than sputum and bronchial washer.

Results of *toxA* gene prevalence revealed that total 19/26(73%) of the isolates were (33) illustrated that this gene was reported in 90.6% of their isolates which is higher than our percentage, while (34) percentage was 89.4%.

Prevalence of toxA in (35) was (33.3%) in the wound and exos was(100%) while the percentage of toxA was (33.3%) in wound and media otitis.

Out of 183 different clinical specimens, 104 that represent (56.8%)*Pseudomonas aeruginosa* isolates were recovered (36).

The genetic typing technic found solutions for the variable phenotype problem and found specific and speed methods for the identification of *P. aeruginosa*. OprL and OprI proteins are found only in this organism they could be a reliable factor for rapid identification of bacterium in clinical isolates.

(38) found percentage of *pseudomonas aerugenosa*as total was (100%) where in burn cases was (33.3%) and in-ear was (13.4%);*P. aeruginosa* owns a variability of virulence factors don't same with or results;*exoS* (86.8%), and were detected (39) that very close to our study.

The burn infection *P. areuginosa* isolates showed high prevalence of virulence factors genes more than wound and pulmonary tract infections isolates as well as the virulence factor gene (ToxA) was show high production in most isolates. In this study we concluded that the virulence factors genes in *P. aeruginosa* is important to human infection especially (ToxA) gene and the PCR technique is very specific and fast method in detection virulence factor genes in *P. areuginosa*(40)(41) and (42).

Where (43)and (44) reach to same results has found percentage of *pseudomonas areugenosa* in wound was (51.4%) and in burn infection was (82.2%) , while prevalence of toxA gene was (75%) while exos gene was (41%) and finally oprL gene was (50%) .

Overall, (95 isolates) *P. aeruginosa* were recovered 34 (35.5%) infected patients were in the age group of 30 - 44 years. There were 24 (25.3%) patients they inside the intensive care unit. A total of 31 (32.6%) strains were isolated from the blood (45).

(33) has found percentage of toxA gene in wound was (90%) that near to our study, while in burn was (97%) that close too, percentage of exos was (62%) in wound that same with us ; and (67%) in burn cases and that very close to as look at table (5).

Percentage of *pseudomonas aureogenosa* that isolated from cow mastitis was (44%) by (46) that same nearly with our percentage.

Different in the values; the prevalence of *pseudomonas areugenosa* and percentage of virulence factors genes depend on several causes including nature of places, immune status of patients, degree of contamination and type and virulence of strain (47).

The results of the current study focused on the percentage of four important

virulence factors in *Pseudomonase aeruginosa* includede xos, toxA, oprL and oprI genes, the results showed more area as percentage of *pseudomonas areugenosa* was in burn infection followed otitis media.

The ToxA gene was represented higher prevalence in wound infection, burn infection, otitis media inflammation and milk samples than others genes, while other genes have different value.

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