Molecular Investigation of Type III Secretion System Toxins-Encoding Genes in Clinical Isolates of *Pseudomonas aeruginosa*

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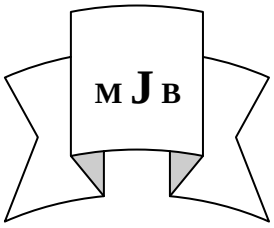

Abstacts

Objectives: This study is aimed to isolate *P.aeruginosa* from different clinical cases and to detect the prevalence of virulence genes encoding type III secretion system toxins in these clinical isolates.

Materials and methods: In this study a total of 422 clinical samples including burn, wound ,ear, urine ,abscess and stool were aseptically taken from out- and inpatients who admitted into two hospitals in Hilla City(Teaching Al-Hilla Hospital and Babylon Hospital for Maternity and children during a period of three months (from March 2011 to June 2011). All samples were subjected to bacterial cultivation for the isolation of *P.aeruginosa*. The isolated *P.aeruginosa* was diagnosed depended on morphological, biochemical and molecular standard characteristics. Type III secretion toxins-encoding genes (*exo*S, *exo*Y, *exo*T and *exo*U) were detected by PCR and the amplification products were separated in 1% agarose gels containing ethidium bromide.

Results: Out of 422 samples, *P.aeruginosa* was isolated from 54 samples(12.8%). The distribution of these isolates were: 22 (55%) from burn samples, 2; (50%) from diabitics foot samples, 8 (14.8%) from wound samples,8 (32%) from ear samples, 3 (11%) from abscess samples,7 (4%) from stool samples, 4 (4%) from urine samples and 0 sputum samples. The genotypic properties of type III secretion system toxins was detected by polymerase chain reaction (PCR). The results of this study revealed that *exo*S gene found in 10/20(50%) of isolates, *exo*Y gene accounted for 12/20(60%) of isolates, while *exoT* detected in most isolates17/20(85%) and *exo*U was detected in minaroty of isolates 5/20(25%).

Conclusion:Type III secretion system toxins-encoding genes found in isolated *P.aeruginosa*, in which *exo*T, *exo*Y and *exo*S gene detected in most isolates while *exo*U gene was detected in minaroty of the isolates.

الخلاصة

هدف الدراسة: تهدف هذه الدراسة الى عزل و تشخيص بكتريا الزوائف الزنجارية من مختلف العينات السريرية و كذلك التحري عن وجود جينات الضراوة المشفرة لسموم نظام الأفراز الثالث في العينات السريرية للبكتريا.

المواد و طرائق العمل: شملت هذه الدراسة جمع ٤٢٢ عينة سريرية تضمنت الحروق، الجروح،الأذن، الأدرار، الخراج والبراز أخذت من المرضى المراجعين و الراقدين في مستشفيين في مدينة الحلة (مستشفى الحلة التعليمي و مستشفى بابل للولادة و الاطفال) خلال فترة ثلاثة أشهر (من شهر آذار ٢٠١١ الى شهر حزيران ٢٠١١). تم زرع جميع العينات في أوساط زرعية مناسبة تحت الظروف الهوائية لغرض عزل الزوائف الزنجارية. شخصت بكتريا الزوائف الزنجارية المعزولة بالأعتماد على الخصائص الشكلية و الكيموحيوية و الجزيئية القياسية . تم الكشف عن الجينات المشفرة لسموم نظام الأفراز الثالث بأستخدام تفاعل البلمرة المتسلسل و نواتج التضاعف فصلت بأستخدام١ ٪ أكاروز يحتوي على صبغة ethidium bromide.

النتائج: اظهرت النتائج أن من مجموع ٤٢٢ عينة، ٥٤ عزلة كانت عائدة للنوع *P.aeruginosa* . وكان توزيع هذه العزلات٢٢ (٥٥٪)عزلة من الحروق، ٢(٥٠ ٪)عزلة من قرحة القدم للمصابين داء السكري ،٨(١٤٪) عزلة من الجروح، ٨(٣٢٪) عزلة من الأذن ، ٣ (١١٪) عزلة من الخراج، ٧ (٤٪) عزلة من البراز ، ٤ (٤٪) عزلة من الإدرار ولم تعزل أي عزلة من القشع. وفي هذه الدراسة تم التحري عن الخصائص الجزيئية لجينات سموم نظام الأفرازالثالث للبكتريا باستخدام تقنية تفاعل البلمرة المتسلسل حيث أظهرت نتائج الدراسة أن جين *exoS* يوجد في (٥٠٪) من العزلات، جين *exoY* تم احتسابه في (٦٠٪) من العزلات ، بينما جين *exoT*وجد في معظم العزلات (٨٥٪) وجين *exoU* وجد في قلة من العزلات (٢٥٪).

الأستنتاج: الجينات المشفرة لسموم نظام الأفراز الثالث وجدت في عزلات بكتريا الزوائف ، جينات *exoS*،*exoT* و*exoY* وجدت في معظم العزلات بينما جين *exoU* وجد في قلة من العزلات.

Introduction

Pesudomonas aeruginosa, a leading gram-negative pathogen causes nosocomial infection has received most attention [1]. It is rarely infects healthy tissues, but, when defenses are compromised, it can infect virtually all tissues [2]. It has the capacity to adapt easily to change in the environment, need minimal nutritional requirement for growth, rapidly develop resistance to antibiotics and produces arsenal of virulence[3]. The pathogenicity of *P. aeruginosa* appears to be related primarily to its toxin repertoire. In gram-negative bacteria have been identified a third(type III) secretion system in which toxins are injected directly into adjacent host cells [4]. There are four known effector proteins are secreted by this system: ExoS, ExoT, ExoU, and ExoY [5]. ExoS and ExoT share 75 % amino acid identity, ExoS and ExoT are bifunctional exotoxins with C-terminal ADP-ribosylation activities., but ExoT possesses a lower catalytic activity, with only 0.2 % of the ADP-ribosyltransferase activity of ExoS [6].ExoU toxin is cytolytic to many mammalian cell types including macrophages, neutrophils, epithelial cells, and fibroblasts. In animal models of acute pneumonia, disruption of the *exoU* gene resulted in decreased virulence, whereas transformation with an *exoU*-expressing plasmid increased the virulence of strains that did not naturally secrete ExoU [7]. ExoY is an extracellular adenylate cyclase which shows a certain homology to extracellular adenylate cyclases of *B.pertussis* and *B.anthracis*[8]. ExoY increases cytosolic cAMP, enhanced by a eukaryotic cofactor This increased cytosolic cAMP leads to increased pulmonary microvascula rintercellular gap formation and increased lung permeability[9]. This study aimed to investigate the prevalence of Type III secretion system toxin genes among clnical isolates of *P.aeruginosa*.

Material and Methods

Specimens

A total of 422 clinical samples were collected from patients at hospital (Al-Hilla Teaching Hospital and Babylon Hospital for Maternity and children during a period of three months (from March 2011 to June 2011) suffering from wounds infections(surgical and burn wound), chronic suppurative otitis media, skin infection(abscess), gastrointestinal(diarrhea) and urinary tract. The samples were immediately inoculated on, MacConkey agar, blood agar and nutrient agar and incubated for overnight at 37°C under aerobic conditions. The bacteria was isolated and identified according to their diagnostic characteristics and then compared with their being reported in referential references Colle*et.al*[10], (1996) and MacFaddin,(2000)[11].

DNA extraction

Chromosomal DNA was extracted according to the genomic DNA purification kits supplemented by manufacturer company(Geneaid,UK).

Detection of type III secretion toxins-encoding genes

Type III secretion toxins-encoding genes (*exo*S, *exo*Y, *exo*T and *exo*U were detected by PCR. Each 20µl of PCR reaction mixture contained 3 µl of upstream primer, 3 µl of downstream primer, 4µl of nuclease free water, 5 µl of DNA extraction and 5 µl of master mix. Thermal cycler conditions are shown in the table-1. The primers of *exo*S, *exo*Y, *exo*T and *exo*U used

generated 565, 749, 152 and 1572bp fragments respectively. The amplification products were separated in 1% agarose gels containing ethidium bromide. DNA ladder 100bp (Geneaid,UK) consist of 12 linear double-stranded DNA fragment with size 100-3,000bp used for compare. After electrophoresis, the gel was photographed under UV light.

Table 1 Primers sequences and thermal cycler conditions

Genes	Primer sequence (5'-3')	Size of product bp	PCR condition	Reference
exo S F *exo* S R	5-TCAGGTACCCGGCATTCACTACGCGG-3 5-TCACTGCAGGTTCGTGACGTCTTTCTTTTA-3	565	94ºC 3min 1x 94ºC 30sec 55ºC 30sec 40x 72ºC 2min 72ºC 5min 1x	Feltman *et.al*.,(2001)[12]
exo Y F *exo* Y R	5-TCCAAGCTTATGCGTATCGACGGTCATC-3 5-CGTATCGATCCGAGGGGGGTGTATCTGACC-3	749	94ºC 3min 1x 94ºC 30sec 55ºC 30sec 40x 72ºC 2min 72ºC 5min 1x	Feltman *et.al.*, (2001)[12]
exo U F *exo* U R	5-AGCGTTAGTGACGTGCG-3 5-GCGCATGGCATCGAGTAACTG-3	1572	94ºC 3min 1x 94ºC 30sec 55ºC 30sec 30x 72ºC 2min 72ºC 5min 1x	Feltman *et.al*.,(2001)[12]
exo T F *exo* T R	5-AATCGCCGTCCAACTGCATGCG-3 5-TGTTCGCCGAGGTACTGCTC-3	152	C 2min 1x 94ºC 30sec 58ºC 30sec 36x 68ºC 1min 68ºC 7min 1x	Ajayi *et.al*., (2003) [13]

Result and Discussion

The results of morphological and biochemical characterization revealed that among the 422 clinical samples, 54(12.8%) isolates were found to be *P. aeruginosa* (table 2). Similar results have been reported by Paramythiotou *et.al*.,(2004) and Boyer *et.al*., (2011) who pointed out that *P.aeruginosa* was isolated in a percentage of (14.1%) and (16%) respectively [14,15].

Table 2 The distribution of *P.aeruginosa* isolates from different clinical samples

Type of Sample.	No. of samples	No. of isolates (%)
Burns	40	22(55%)
Wounds	54	8(14.8%)
Diabetics' foot	4	2(50%)
Ear	25	8(32%)
Stool	150	7(4%)
Urine	92	4(4%)
Abscess	27	3(11%)
Sputum	30	0(0.0%)
Total No.(%)	422	54(12.8%)

Results of the present study indicated that *P.aeruginosa* infections mainly affected the burn patients in which,the highest *P.aeruginosa* isolation rate 22/40(55%) was obtained from burn samples followed by diabitics foot2/4(50%),wound 8/54 (14.8%), ear 8/25(32%), abscess 3/27(11%), stool7/150(4%), urine 4/94 (4%) and sputum 0. These results were in agreement with that reported by local and global studies. Al-Mashhadani,(2004) reported that *P. aeruginosa* represent (32.69%), (23%) and (3.85%) of wound and burn, respiratory and ear infection and present in (1.92%) of stool samples[16]. (Saleh ,2007) found that *P. aeruginosa* was responsible for (32.69%), (21.4%) and (20%) of burn, wound and chronic otitis media[17]. In a study conducted by Gad *et.al*., (2007), *P. aeruginosa* was isolated at a percentage of (72%), (11%), (20%),(20%),(22%) and (4.4%) from burns, wounds, ear, abscess, urine and stool samples respectively[18]. The likelihood of recovery from *Pseudomonas* infection is related to the immune state of the patients and severity of the patient's underlying disease process [19].

Detection of type III Secretion system toxins genes

A total of 20 isolates of *P.aeruginosa* obtained from different of clinical source were subjected to molecular studies by PCR. These include 5 isolates from burns, 2 isolates from wounds, 2 isolates from diabitics foot, 2 isolates from ear, 2 isolates from abscess , 3 isolates from urine and 2 isolates from stool. Specific PCR primers were used for detection of (*exo*S) gene as shown in figure(1). The results showed that overall, 10/20(50%) of investigated isolates contained the *exo*S gene. The results are in agreement with those results obtained by Finlayson and Brown (2011), who reported that (51%) of *P.aeruginosa* isolated from variety of clinical sources contain this toxin[20]. In general surveys for

proportion of strains carrying of T_3SS genes, it was found that (*exo*S) gene was common [21]. Invasive strains of bacteria, bear the gene for exoenzyme S [22]. The ability to secrete exotoxin S facilitates invasion and internalization. It contributes to dissemination in a burn model and to pathology

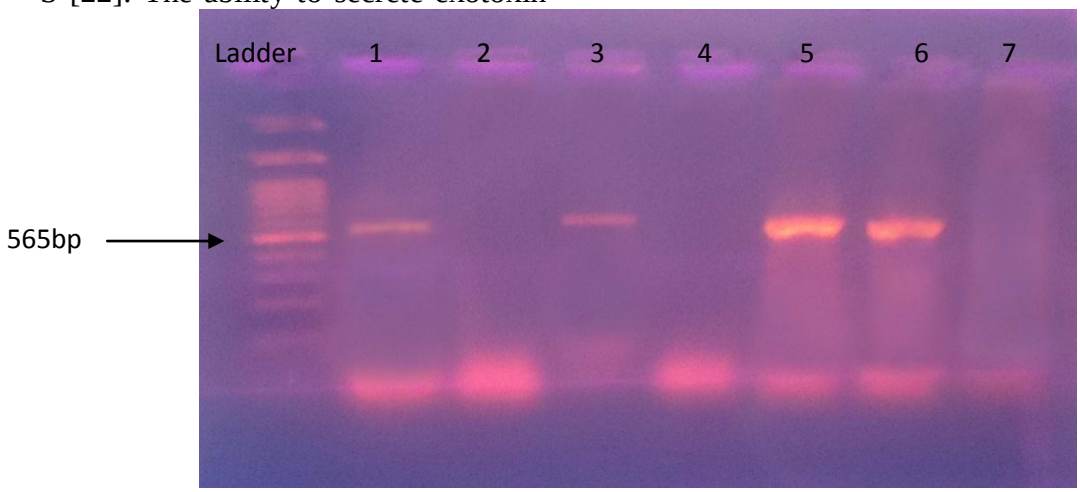

Figure 1 Gel electrophoresis of PCR product of exotoxin S gene(*exo*S)
Lane of the isolates numbered (1,3,5,6) were positive for (*exo*S),whereas isolates numbered (2,4,7,) were negative for (*exo*S)

in a lung model of disease.The N terminus of toxin encodes GTPase-activity that inactivates Rho GTPases, leading to cytoskeletal alterations. GAP activity has been shown to play a major role in blocking internalization of bacteria by both phagocytic and nonphagocytic cells. The C terminus of toxin encodes ADP-ribosyltransferase (ADPRT) activity, which has numerous deleterious effects on host cells in vitro, including induction of long-term cell rounding, as well as decreased DNA synthesis, and cellular adherence[23].Host target proteins modified by exotoxin S ADP-ribosyltransferase activity include members of the Ras family of proteins, vimentin, the Fc region of IgG3, and apolipoprotein[24].

The molecular detection of *exo*Y gene is illustrated in figure(2). The results indicates that 12/20(60%) of isolates possess this gene. Other investigators reported that the most clinical isolates of *P.aeruginosa* have this gene [27][12].**Winstanley** *et.al*., (2005) noted that (81%) of examined corneal isolates contained the toxin. Exotoxin Y is more recently discovered an adenylate cyclase [25]. Injection of *exoY* into mammalian cells results in an elevation of intracellular cAMP concentration and differential expression of multiple genes including many known to be regulated by cAMP. This leads to disruption of the actin cytoskeleton, inhibition of bacterial uptake by host cells and increased endothelial permeability, Such activities would be predicted to lead to more severe disease[26].

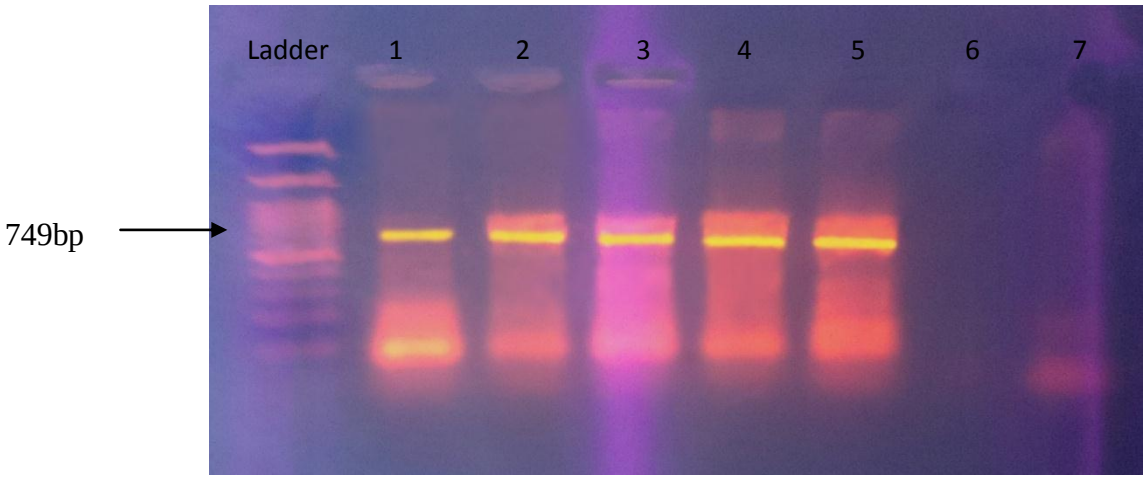

Figure 2 Gel electrophoresis of PCR product of exotoxin Y gene (*exo*Y)

Lane of the isolates numbered (1,2,3,4,5) were positive for (*exo*Y), whereas isolates numbered (6,7) were negative for (*exo*Y)

Primers of *exo*T gene used for detecting the presence of *exo*T gene in *P.aeruginosa* isolates, Figure (3) shows positive amplification for this gene. Relatively high prevalence 17/20(85%) of this toxin among *P.aeruginosa* isolates seen in this study. Hauser, (2009) stated that *exo*T gene present in 91-100% of clinical isolates taken from acute infections[28]. In addition, Feltman *et.al.*, (2001) and Fleiszing *et.al.*,(1997) referred that *exo*T gene found in all (clinical and environmental) *P.aeruginosa* isolates, suggested that this gene is not variable trait[12][27]. Bradbury *et.al.*, (2010) reported that a small number of individuals *P.aeruginosa* isolates have showed deletions of some of the specific tested virulence factor genes including *exo*T gene[28]. Toxin T is the only type III effective protein reported to date that is encoded by both invasive and cytotoxic *P. aeruginosa* strains. This gene found commonly across strains would suggest an important role in survival; this may involve the control of phagocytosis by eukaryotic cells [29].

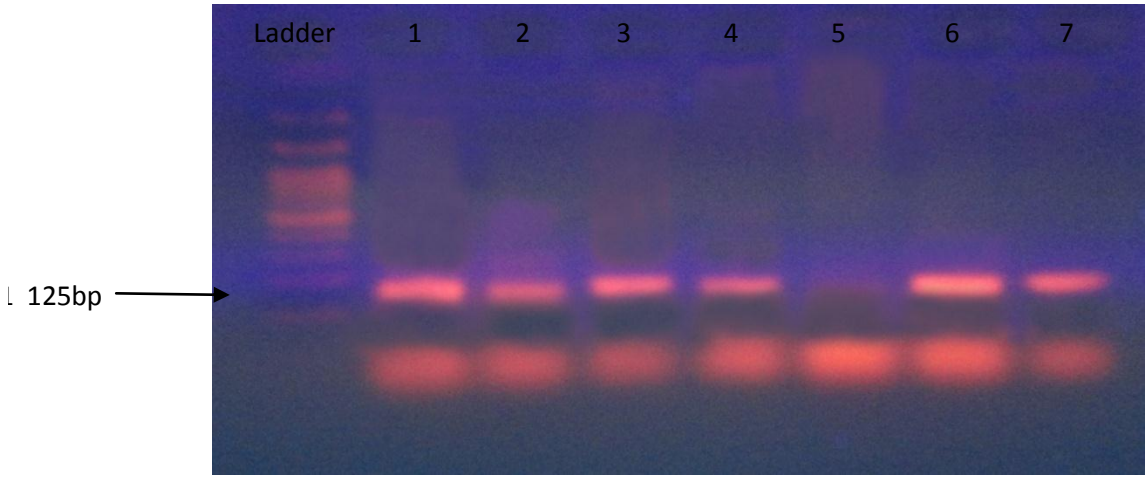

Figure 3 Gel electrophoresis of PCR product of exotoxin T gene (*exoT*) gene

The isolates numbered (1,2,3,4,6,7) were positive for (*exo*T), whereas isolates numbered were (5) negative for (*exo*T)

Exotoxin U gene (*exo*U) was investigated by PCR technique using specific primers for this gene, since positive amplification was seen for this gene as shown in figure (4). This gene encodes for exotoxin U found in minority among *P.aeruginosa* isolates, that 5/20(25%) of isolates were found to posses this gene. These result are in accordance with those results being reported by Wong-Beringer *et.al*.,(2008) who indicated that this gene accounted for (27%) in *P.aeruginosa* isolates[30].

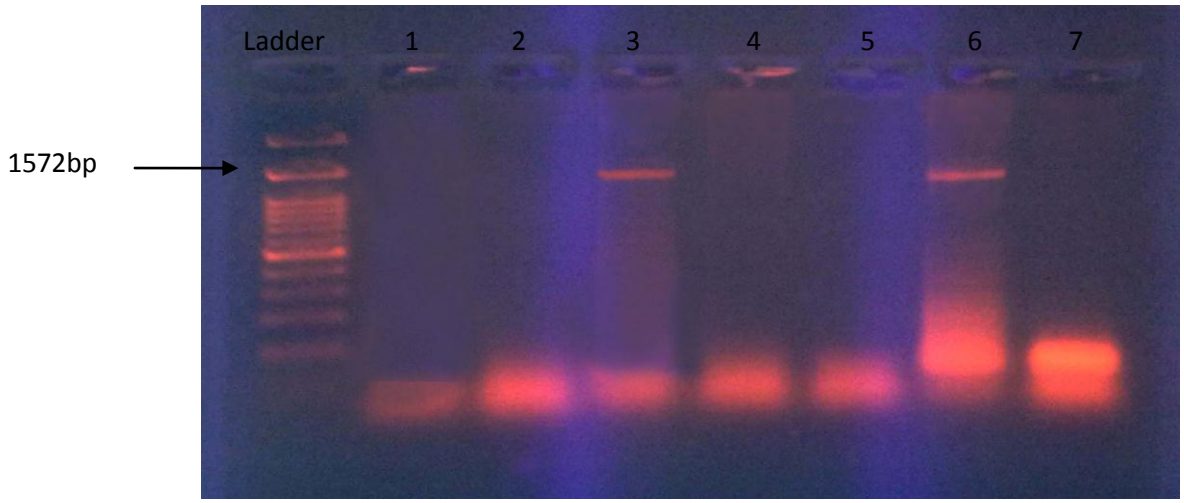

Figure 4 Gel electrophoresis of PCR product of exotoxin U gene(*exoU*)

The isolates numbered (3,6) were positive for (*exo*U),whereas isolates numbered (1,2,4,5,7) were negative for (*exo*U)

Other research suggested that prevalence of *exo*U gene in acute infection caused by *P.aeruginosa* range from 28 to 42% [30].The *exo*U gene is present in approximately one-third of isolates obtained from acute infections, and secretion of the *exo*U toxin is a marker for strains with enhanced virulence [31], *exo*U, like many of the genes that encode type III secretion effectors proteins, may have been acquired by *P. aeruginosa* through horizontal transfer from another bacterial species [32]. Comparison of genomes of various *P. aeruginosa* strains showed that the *exo*U determinant is found in the same polymorphic region of the chromosome near a Trna(Lys) gene, suggesting that *exo*U is a horizontally acquired virulence determinant. The evolutionary history very likely involved transposition of the *exo*U determinant onto a transmissible plasmid, followed by transfer of the plasmid into different *P. aeruginosa* strains, the plasmid subsequently integrated into a Trna(Lys) gene in the chromosome of each recipient, where it acquired insertion sequences and underwent deletions and rearrangements [33]. The secretion of this toxin, blocks phagocyte-mediated clearance at the site of infection and facilitates the establishment of an infection. This result is a local disruption of an essential component of the innate immune response [34]**.** It is

known that 90% of *exo*U-producing *P.aeruginosa* strains are associated with severe infection [3]. In vitro, *exo*U rapidly kills a number of different cell types, including epithelial cells, macrophages, and fibroblasts [27]. ExoU as a phospholipase is consistent with the observed loss of cytoplasmic contents and membrane permeability in mammalian cells and the vacuolar fragmentation. Lipase activity of this toxin appears to have broad substrate specificity that includes the hydrolysis of neutral lipids, presumably diacylglycerols and triacylglycerols as well as phospholipids [26].

Thus from the results expressed above, *P.aeruginosa* was common bacteria causes nosocomial and community infections. Type III secretion system toxins-encoding genes were not found in all *P.aeruginosa* isolates. Most *P.aeruginosa* isolates had at least two genes of these toxins.

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