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Molecular and Phenotypic Study of Virulence Genes in a Pathogenic Strain of *Pseudomonas aeruginosa* isolated from various clinical origins by PCR: Profiles of genes and Toxins.

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ABSTRACT

Most infections associated with bacteria like *Pseudomonas Aeruginosa* owns a variability virulence factors elements which can increase bacterial pathogenicity and infection severity. This study was aimed to revealed pathogenic genes which are related to bacterial virulence by PCR technique of *P. aeruginosa* isolated from various clinical cases, with the aim to discover the connection of these pathogenic elements related to special *P. Aeruginosa* infections. Strains of *P. aeruginosa* (n = 286) were gathered in time between April 2014 and April 2015 at the medical Laboratory, private clinic laboratory and burning wards at Educational Hospital at Babil province then transport specimens for cultivating and identification. DNA (Plasmid) disjointed and isolated using standard distinct methods. Several structural and virulence genes of Ps. aeruginosa including (*plcH*, *algD*, *rhlI*, *exoS*, *exoU*, *lasR*, *toxA*, *aprA*, *rhlAB*, *fliC*, *lecA*, *toxR*, *lasI*, *oprI*, *oprL*, *rhlR*, *nan1*, *lasB*) were amplified using the PCR technique by expending precise primers designed by using Primer3Plus, PCR condition and sequencing of each primer pair. Ps. aeruginosa own genes that have the ability for encoding: *plcH, algD, rhlI, exoS, exoU*, *lasR, toxA, aprA, rhlAB, fliC, lecA, toxR, lasI, oprI, oprL, rhlR, nan1, lasB*. Virulence genes prevalence among *P. aeruginosa* isolates (n=286) were as follows: *lasI* 3.5%, *lasR* 2.0%, *rhlI*, 2.4%, *rhlR* 4.3%, *toxA* 9.9%, *aprA* 2.1%, *rhlAB* 2.6%, *plcH* 10.5%, *lasB* 10.6%, *fliC* 2.5%, *lecA* 4.7%, *algR* 10.4%, *toxR* 4.7%, *oprI* 6.4%, *oprL* 7.5%, *nan1* 2.0%, *exoS* 9.4%, *exoU* 4.5%. Blood infections revealed the highest ratio in virulence genes from all infection 24 (20.9 %), followed Burn infections 86 (17.4%), UTI 92 (16.6%), Wound 33 (15.5%), LRTI 16 and URTI 35 (15%).

Keywords: *Pseudomonas aeruginosa*, virulence genes, PCR, Plasmid Curing.

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INTRODUCTION

Pseudomonas Aeruginosa infections deliberated as multifactorial, as proposed by the enormous amount of extracellular virulence and cell-associated factors furthermore it consider as an opportunistic pathogen capable of infecting virtually all tissues[1]. At the beginning *Ps. aeruginosa* infections colonize on the altered epithelium. Adherence on epithelium was intervened by many factors like pili, fimbriae and flagella. Production of numerous extracellular virulence factors occurred after colonization and these factors blamable for dissemination, invasion bloodstream, and wide-ranging tissue damaging [2].

From these virulence factors there are many important toxins and enzymes like exotoxin A, exoenzyme S, sialidase and elastase which are compactly controlled by cell signaling systems [3]. Exotoxin A and exoenzyme S which secreted by a type III section system was repressed by Protein biosynthesis [4]. Las B (zinc metalloprotease) has activity as an elastolytic on lung tissue. Sialidase which responsible for adherence to the respiratory tract encoded by nan1 gene [5].

Guiding systems prepare Pseudomonas to manufacture their virulence elements in a coordinated, cell mass reliant on style that can permit *Ps. Aeruginosa* to beat the mechanisms of host defense. Intervention through all these virulence elements assembly in need the system in these cells called cell signaling which is an auspicious therapeutic methodology to reducing the percentage of mortality and morbidity that triggered by *Ps. Aeruginosa* [6, 7].

Colonization with mucoid *P. aeruginosa* on pulmonary tract was a main cause of infection cases in patients with cystic fibrosis [8]. *Ps. Aeruginosa* infections mainly distress in many patients with chronic illnesses, catheterization and burn in addition to many other infections [9]. Fast recognition of isolates of causative agents is actually essential for subsequent treatment choice for patients. Polymerase Chain Reaction "PCR" was essential for recognizing etiological genus quickly by magnification the unique series of nucleotide (sequence) to a specific being [10]. Lipoproteins"I, L" are external proteins forming membrane of *Ps. Aeruginosa* which is blamable for resistance of Pseudomonas to antibiotics. Because of these proteins are originate merely in this bacteria, it can be useful and dependable aspect for fast identification of *P. Aeruginosa* [11, 12].

The aim of our study was to estimate prospective relations concerning the incidence all alleged genes which correlated and vital for virulence mechanism and the consequence of toxicities affected by bacteria in addition to that it was attempt to distinguish the molecular indications of virulence for *Ps. aeruginosa* strains that isolated from different medicalorigins [7].

METHODS

*P. aeruginosa*isolate and identification

Strains of *Ps. aeruginosa*(*n* = 286) were gathered in time between April 2014 and April 2015 at the medical Laboratory, private clinic laboratory and burning wards at Educational Hospital at Babil province then transport specimens for cultivating and identification. Totally isolates were confirmed cultural procedures in addition to detect soluble virulence factors produced by *Ps. aeruginosa* strains like; Hemolysins, DNase Lecithinase , Amylase and Lipase and then using vitek 2 compact for microbiological revealing (BioMérieux, France) which circling more than 30 biochemical tests using fluorescent technique, containing manytests basically depending on enzymatic reaction for oxidases and amino peptidases. The source of the isolates was from different systemic infection sites of clinically ill patients of mentioned Hospital. All these strains were analyzed for virulence gene content and for the correlation of certain genes or gene combinations with known chromosomal genes.

Preparation of Plasmid DNA

DNA (Plasmid) disjointed and isolated using standard distinct previously [13]. Plasmid profiling were built by combination of many strains which having the similar molecular and quantity of a profile organizing a main profile.

DNA extraction

A total of 286 isolates of Ps. aeruginosa that cultivate aerobically in brain heart broth and incubated for 18–24 hour at 37°C and 1200 rpm in a shaker incubator, the bacterial DNA extracted using commercial DNA extraction kit it was performed using Invitrogen DNA extraction kit (USA) and then the genomic DNA was conserved at -80°C in deep freezer.

Curing of Plasmid

By using technique for curing of the resistant plasmids from medical isolates belong to Ps. aeruginosa we acquisition the pure and highly quantitative / qualitative plasmid [14]. The extraction of plasmid done by using Gene aid Kit and follow the manufacture procedure.

Virulence Genes Detection

Several structural and virulence genes of Ps. aeruginosa including (*plcH, algD, rhlI, exoS, exoU*, *lasR, toxA, aprA, rhlAB, fliC, lecA, toxR, lasI, oprI, oprL, rhlR, nan1, lasB*)were amplified using the PCR technique by expending precise primers designed by using Primer3Plus, PCR condition and sequencing of each primer pair was showed in Table 1.

Table 1: *Pseudomonas Aeruginosa* Primers set of virulence genes used in this study

Gene	Forward	Reverse	Tm	bp.
lasI	5’ CGTGCTCAAGTGTTCAAGG 3’	5’ TACAGTCGGAAAAGCCCAG 3’	66	295
lasR	5’ AAGTGGAAAATTGGAGTGGAG 3’	5’ GTAGTTGCCGACGACGATGAAG 3’	66	130
rhlI	5’ TTCATCCTCCTTTAGTCTTCCC 3’	5’ TTCCAGCGATTCAGAGAGC 3’	60	155
rhlR	5’ TCAGGGCGCACGAGAGCAACGAGA 3’	5’ CACTTCCTTTTCCAGGACG 3’	59	133
toxA	5’ GGAGCGCAACTATCCCACT 3’	5’ GACAGCCGCGCCGCCAGGTAGAGG 3’	66	454
aprA	5’ GTCGACCAGGCGGCGGAGCAGATA 3’	5’ GCCGAGGCCGCCGTAGAGGATGTC 3’	62	994
rhlAB	5’ TCATGGAATTGTCACAACCGC 3’	5’ ATACGGCAAAATCATGGCAAC 3’	61	151
plcH	5’ GAAGCCATGGGCTACTTCAA 3’	5’ AGAGTGACGAGGAGCGGTAG 3’	66	307
lasB	5’ TTCTACCCGAAGGACTGATAC 3’	5’ AACACCCATGATCGCAAC 3’	65	153
fliC	5’ GGCAGCTGGTTNGCCTG 3’	5’ GGCCTGCAGATCNCCAA 3’	60	1025
lecA	5’CGATGTCATTACCATCGTCG3’	5’TGATTGCACCCTGGACATTA3’	65	215
algD	5’AGGGCAACTGGACGGCTATC3’	5’TGTGGTCGGCAATGAAGAAGA3’	63	437
toxR	5’ATGGCATCTATGCGAGGAAC3’	5’GCAGGGGAATGAAGTTCTTG3’	65	207
oprI	5’ATGAACAACGTTCTGAAATTCTCTGCT3’	5’ CTTGCGGCTGGCTTTTTCCAG3’	57	249
oprL	5’ATGGAAATGCTGAAATTCGGC3’	5’ CTTCTTCAGCTCGACGCGACG3’	57	504
nan1	5’AGGATGAATACTTATTTTGAT3’	5’ TCACTAAATCCATCTCTGACCCGATA3’	55	1316
exoS	5’ CTTGAAGGGACTCGACAAGG3’	5’TTCAGGTCCGCGTAGTGAAT3’	54	504
exoU	5’GGG AAT ACT TTC CGG GAA GTT3’	5’CGA TCT CGC TGC TAA TGT GTT3’	60	428

Set up of PCR mixture reaction

The PCR was performed in 20µl reaction mixtures inclosing DNA template (bacterial DNA) of 1.2µl, 1µl of 25 mM MgCl2, 5µl of 5x reaction buffer, 0.5 µl concentration of each (dNTP) deoxynucleotide triphosphate, 1.5µl of each forward primer and reverse primer and 0.15µl DNA polymerase along with its amplification buffer. Gene magnifications done using convention Veriti gradient thermal cycler from applied biosystems (USA).

Antibiogram Test

Disk diffusion (Kirby-Bauer method) achieved to determine bacterial susceptibility and sensitivity. Antibiotics tested were tested against Ps. aeruginosa Amikacin, Amoxicillin, Amoxicillin + Clavulanic acid, Azithromycin, , B \Bacitracin, Carbenicillin, Cefodizime, Cefoxitin, Ceftizoxime, Cephalexin, Chloromphenicol, Clarithromycin, Clindamycin, Erythromycin, Gentamycin, Kanamycin, Lincomycin, Methicillin, Nitrofurantoin, Norfloxacin, Ofloxacin, Oxacillin, Oxytetracyclin, Penicillin G, Piperacillin, Rifampim. Results were recited in agreement with Clinical and Laboratory Standards Institute (CLSI, 2009). Results was revealed in figure 3.

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RESULTS AND DISSCUSION

Amplification of bacterial genome done by using adequate assays by PCR technique toreveal Ps. aeruginosa virulence genes, it was many and that associated with their pathogenicity. Some of virulence factors assistance bacterial establishment and colonization on the surface of the host, while others expedite invasion numerous tissue. Many factors influence bacterial colonization such as flagella, fimbriae, polysaccharides surface and type IV pili all these elements considered essential for attachment mechanism. Ps. Aeruginosa have the ability to invade tissues with assistance toxins and enzymes that disruption fleshly barriers by disturbing membranes of the cells and defeating the host, besides the fighting against phagocytosis and immune shield of the host.

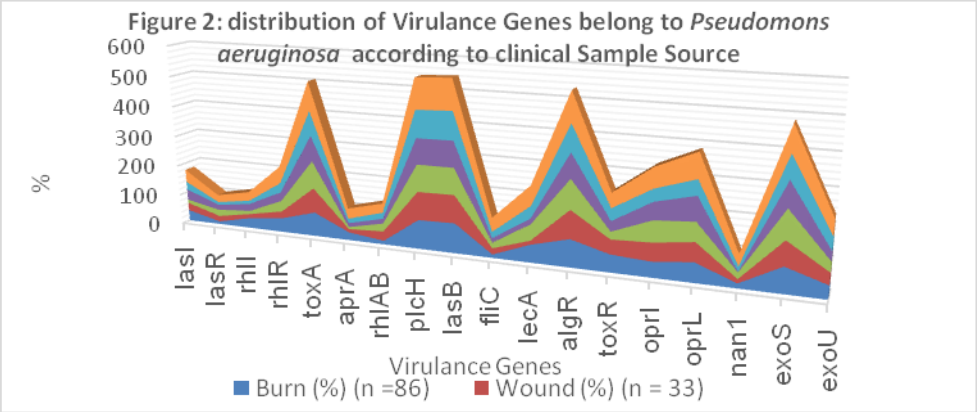
The invasion by Ps. aeruginosa is endorsed by manufacture many virulence proteins like; leucocidin, hemolysins and proteases. Ps. aeruginosa have the ability to produce numerous proteases like alkaline protease LasB and protease IV LasA which caused complement system disruption in addition to degradation of surfactant [15].

Innate immunity can be disrupted by Ps. aeruginosa proteins through inactivation TNF and cleavage antibodies [15]. Elastin was the major component of lung tissue which in charge for lung enlargement and reduction and lying blood vessels, which play a role in their elasticity. The concentrated action of protease (LasB and LasA) is accountable for many pathogenic activity such as demolition of elastin and elastolytic action on human [16].

virulence genes frequency that code for Transcriptional regulator (*lasR*), Exotoxin A (*toxA*), elastase (lasB), alkaline metalloproteinase (*aprA*), Transcriptional regulator (*rhlR*), rhamnolipid (rhlAB), *LecA* protein, auto inducer synthesis protein (*rhlI*), alginate (algD), hemolytic phospholipase C (plcH), flagellar filament structural protein (*fliC*), auto inducer synthesis protein (*lasI*), , transcriptional regulator (*toxR*), outer membrane lipoprotein (*oprI*), peptidoglycan-associated lipoprotein *(oprL*), exoenzyme S (*exoS*) was resoluteusing PCR technique.

Pseudomonas aeruginosa possess a number of genes as virulence elements which used it for attachment, colonization, terminate, and extent through host organs and tissue. This study confirmed that all studied Ps. aeruginosa strains have numerous virulence elements, several of these elements which are terminated, accountable for the many medical cases and accelerate processes of infection which induced through infectious mediators. Ps. aeruginosa own genes that have the ability for encoding: *plcH, algD, rhlI, exoS, exoU*, *lasR, toxA, aprA, rhlAB, fliC, lecA, toxR, lasI, oprI, oprL, rhlR, nan1, lasB*.

Virulence genes prevalence among *P. aeruginosa* isolates (n=286) were as follows: *lasI*3.5%, *lasR*2.0%, *rhlI*, 2.4%, *rhlR* 4.3%, *toxA*9.9%, *aprA*2.1%, *rhlAB*2.6%, *plcH*10.5%, *lasB*10.6%, *fliC*2.5%, *lecA*4.7%, *algR*10.4%, *toxR*4.7%, *oprI*6.4%, *oprL*7.5%, *nan1*2.0%, *exoS*9.4%, *exoU*4.5% as showed in figure 2.



Blood infections revealed the highest ratio in virulence genes from all infection 24 (20.9 %), followed Burn infections 86 (17.4%), UTI 92 (16.6%), Wound 33 (15.5%), LRTI 16 and URTI 35 (15%) as showed in figure 1.

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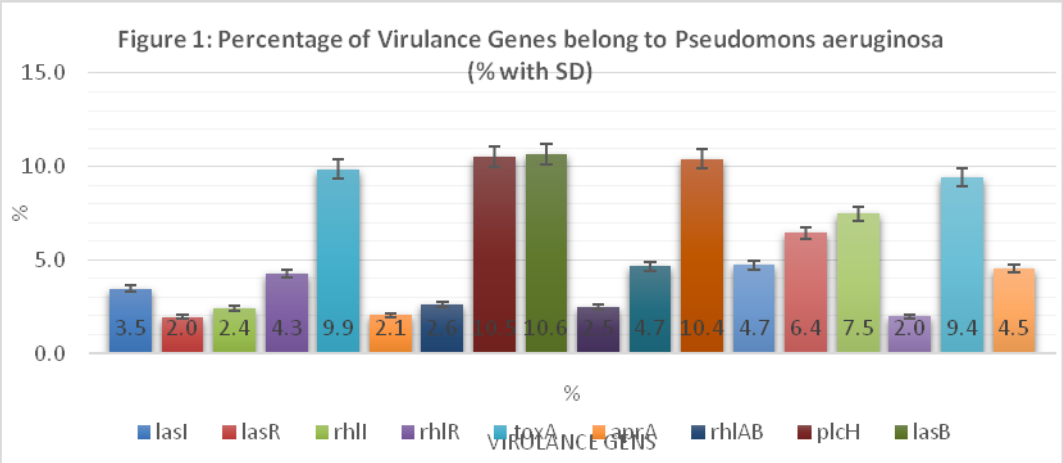
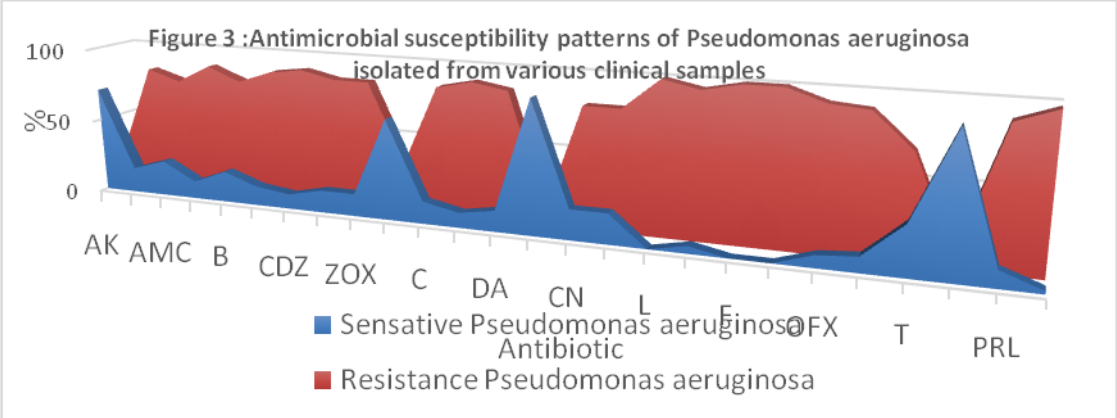


Pathogenicity of bacteria can be induced by the existence of several virulence elements which encoded through sets of genes existent on pathogenicity islands inside bacterial chromosome following cooperate various mixtures [17, 18, 19]. Our study display that entirely virulence genes existing in all isolates and custom part of genome of *P. aeruginosa*. That results agreed with other studies which proposed that the virulence genes sheltered in all strains of *Pseudomonas* individually from the positions of clinical sample [20, 21].

The prevalence of extracellular genes that responsible for encoding many virulence elements like lasB (elastase), aprA (alkaline protease), TCF (protease IV) and many other genes could be revealed using PCR technique as golden standard also other soluble proteins complicated in invasion factor of Ps. aeruginosa are characterized by phospholipases C and rhamno lipid additionally to other virulence elements. All these factors could turn together to interruption tissue components like; phospholipids, in addition associate invasion using their effects (cytotoxic) on WBC especially on lymphocytes, neutrophils and other cells[22].The percentage of *P. aeruginosa* isolates producing the virulence factors from different clinical sources was showed in figure 4.



Many of these proteins which play as virulence factors play essential role in host immunity. For example, Protease consider as essential virulence element in *P. Aeruginosa* pathogenesis because their role in prompted bacterial keratitis [23]. Protease IV virulence role in pathogenicity was recognized by their role in host proteins destruction, moreover destroys of structural proteins helping microbial attachment then causing infection. Also Elastin is a main component of many essential organs in human like blood vessels and lung which in control for lung expansion and contraction and resilience vessels. The intensive action of LasB and LasA protease is elastolytic activity which associated destruction of elastin in tissue and the when invasive by Ps. aeruginosa. Elastase like Zinc metallo protease (LasB) that related to gene operon of lasB, which responsible of many disturbance effects like collagen, elastin and fibrin breakdown [24].

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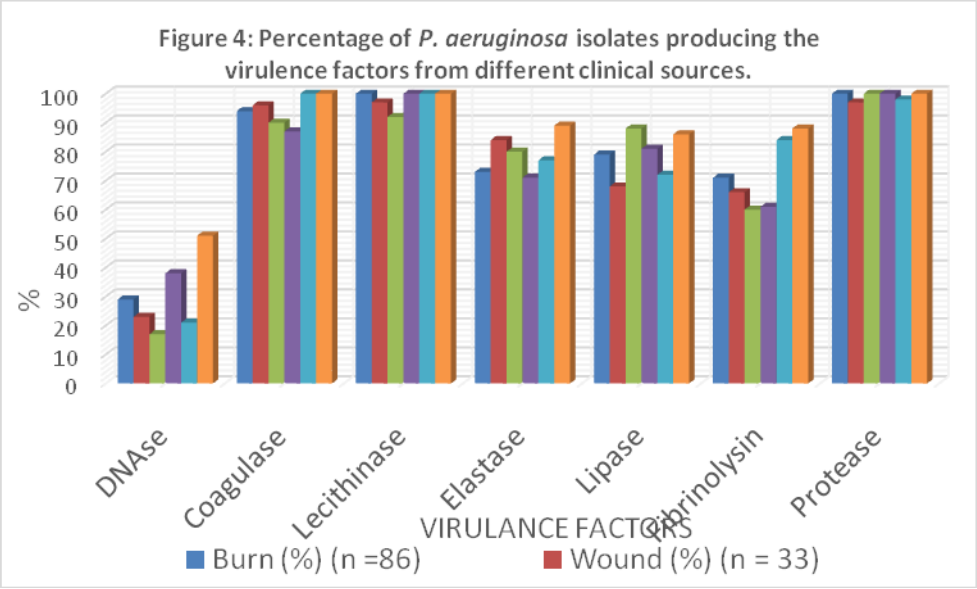

The anibiograms study of *P. Aeruginosa* strains from different clinical origins is revealed in figure 3. Selected strains of *P. Aeruginosa* bared sensitivity to Erythromycin, Amikacin and Penicillin where as presented resistance to Ofloxacin, Kanamycin, Penicillin, Clindamycin, Rifampim, Lincomycin, Oxytetracyclin G, Carbenicillin, Piperacillin, erythromycin, Ceftizoxime, Oxacillin, Nitrofurantoin, Norfloxacin, Amoxicillin, Cephalexin, Methicillin, Chloromphenicol, Amoxicillin/Clavulanic acid, Azithromycin, Norfloxacin, Bacitracin, Cefodizime, Cefoxitin, Clarithromycin and Gentamycin.

Figures from **A – E**was Ethidium bromide-stained Agarose Gel Electrophoresis of PCR-amplified products from extracted (1.5%) patterns showing typical PCR amplification products in multiplex PCRs for all figures including ***plcH, algD, rhlI, exoS, exoU, lasR, toxA, aprA, rhlAB, fliC, lecA, toxR, lasI, oprI, oprL, rhlR, nan1***and***lasB*** genes. Lane L, was DNA ladder (bioneer 25/100 Mixed DNA ladder and 100 DNA ladder)

Figure A: showed *exoU* gene (428 bp.) and *lasI* gene (295 bp.) at the right side of Ladder and in the left side of Ladder it was *fliC* gene (1025 bp.) and *oprL* gene (504 bp.) with 100 DNA ladder.

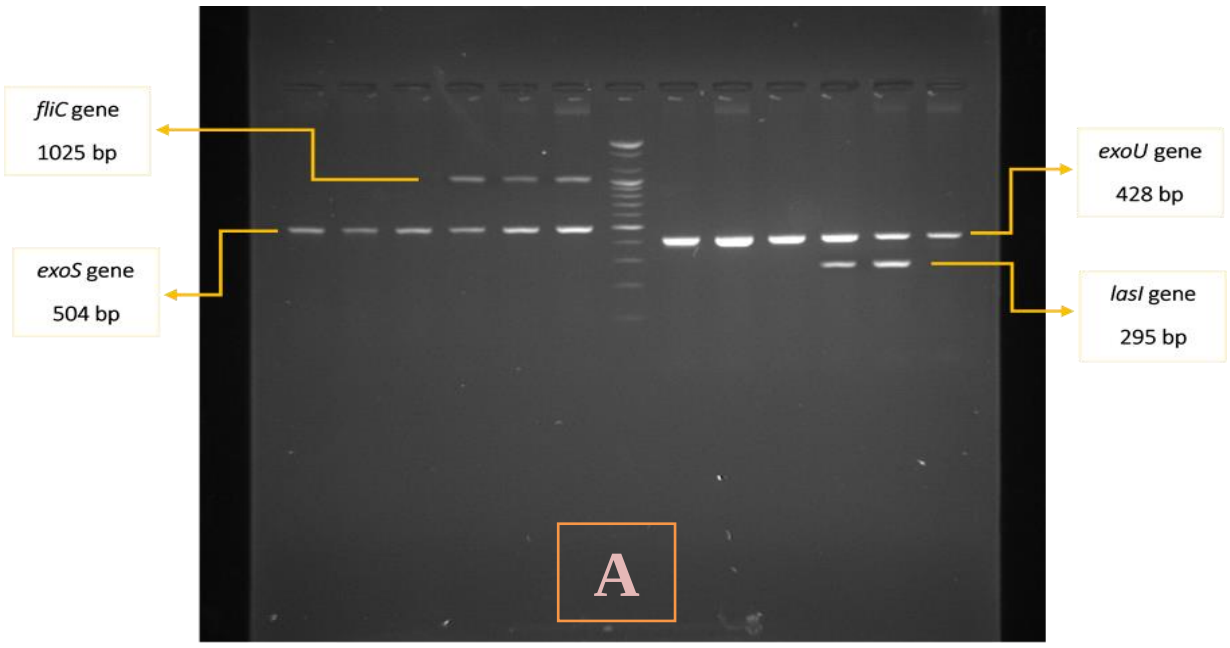

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Figure B: showed *aprA* gene (994 bp.) and *oprL* gene (249 bp.) at the right side of Ladder and in the left side of Ladder it was *nan1* gene (1316 bp.) and *exoS* gene (504 bp.) with 100 DNA ladder.

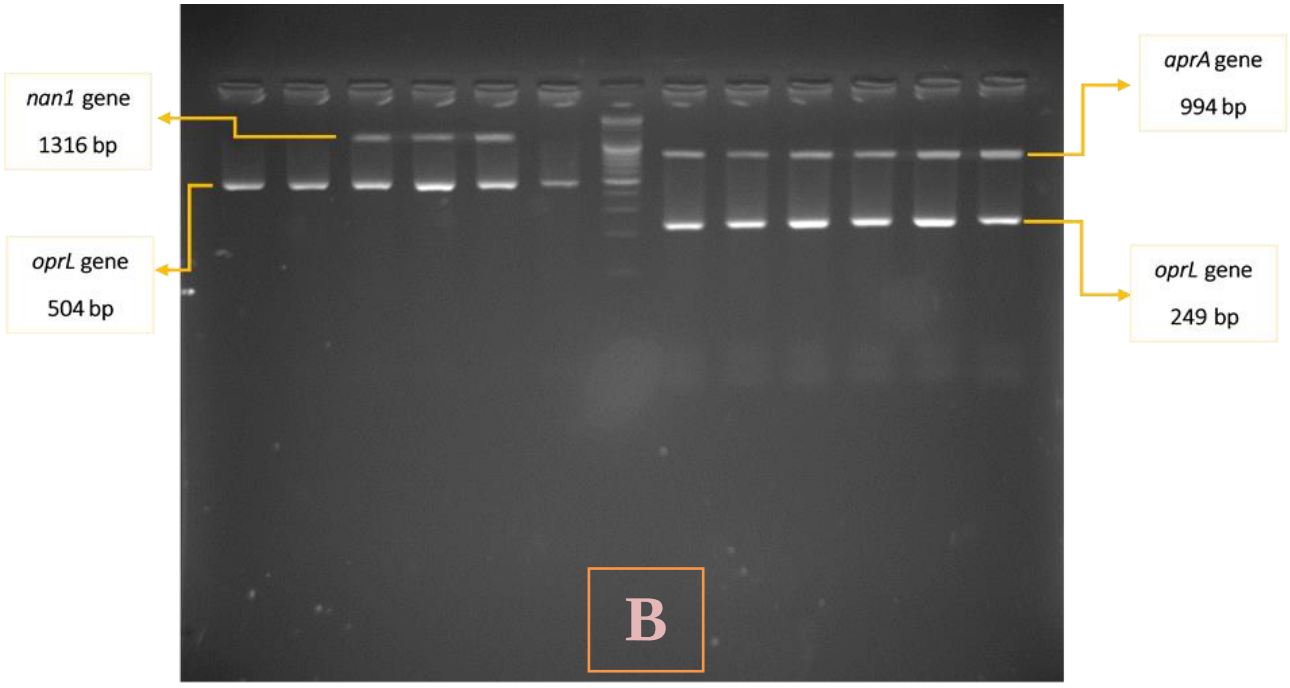

Figure C: showed *rhlAB* gene (151 bp.) and *rhlR* gene (133 bp.) at the right side of Ladder and in the left side of Ladder it was *rhlI* gene (155 bp.) and *lsaR* gene (130 bp.) with 100 DNA ladder.

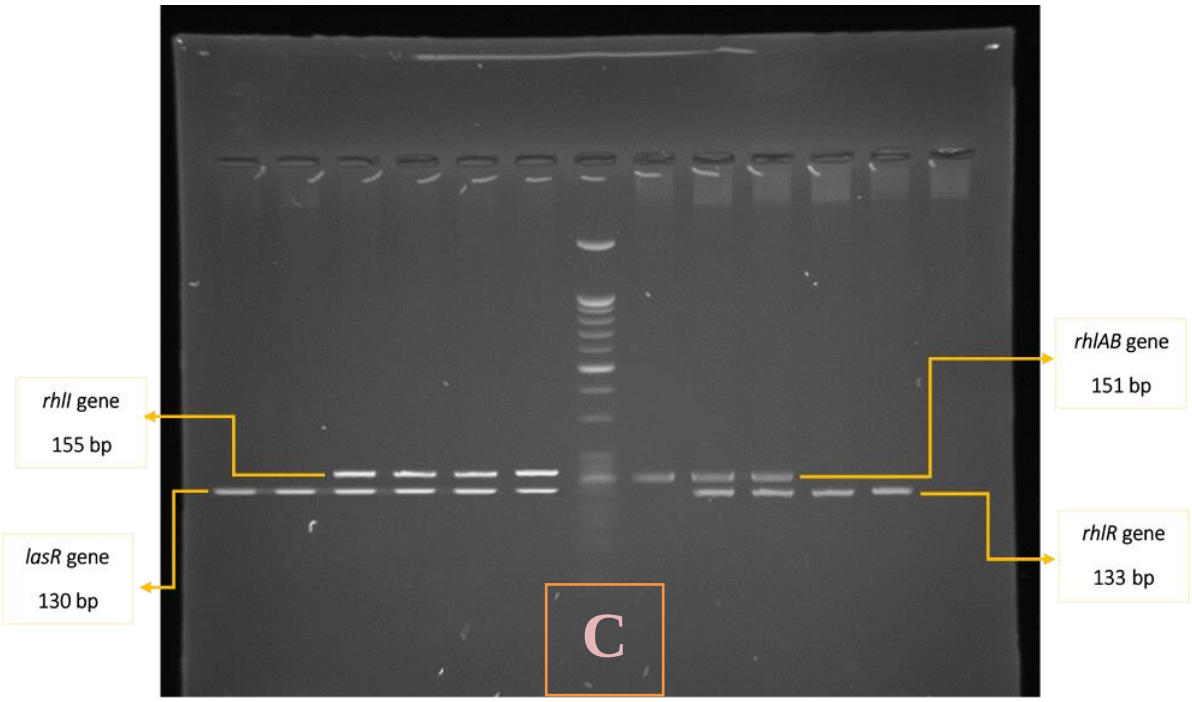

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Figure D: showed *toxA* gene (454 bp.) and *lecA* gene (215 bp) with with 100 DNA ladder.

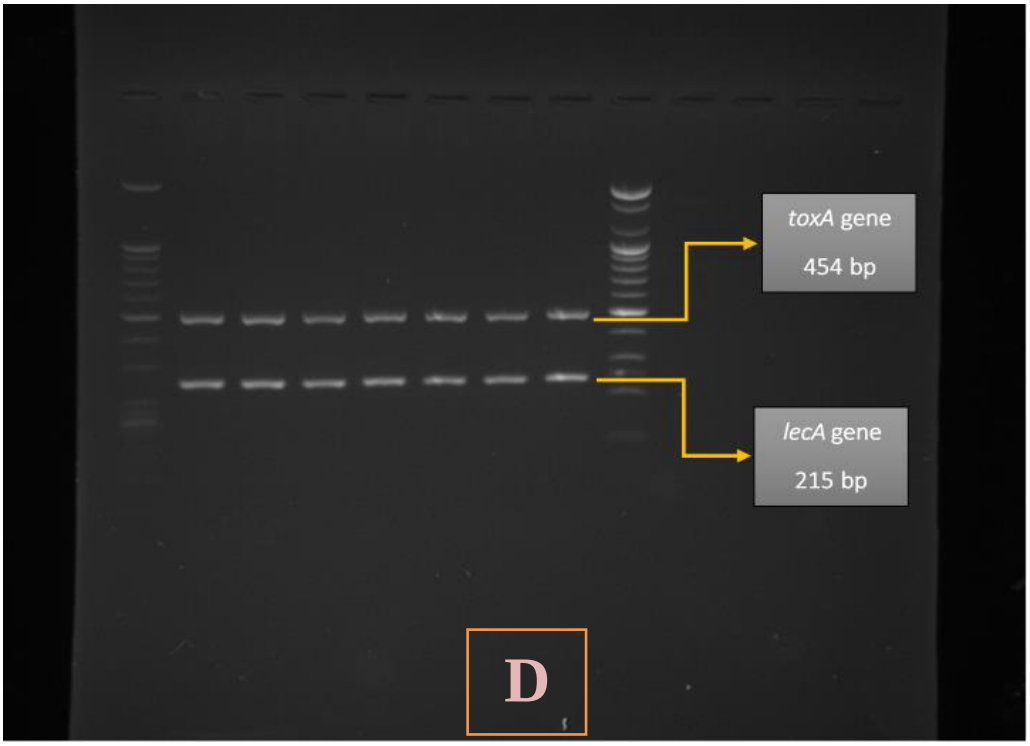
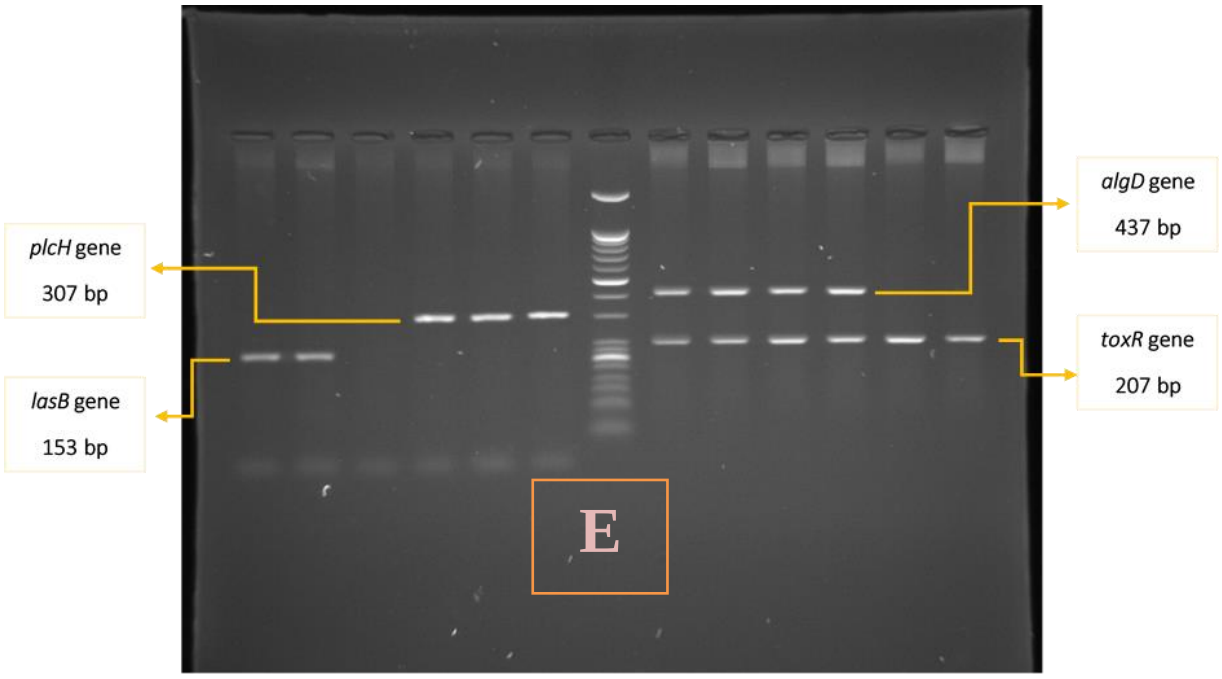


Figure E: showed *algD* gene (437 bp.) and *toxR* gene (207 bp.) at the right side of Ladder and in the left side of Ladder it was *plcH* gene (307 bp.) and *lasB* gene (153 bp.)with 25/100 Mixed DNA ladder.



Aggregate conflict to many antibiotics like fluroquinolone which occurs in several hospitals, that realistic usage is both expelled and limited to get the emerging resistance under control [25, 26]. Resistance to Cefodizime was described as 18%, in this study it was more than 90%. High standards of resistance which detected comparable to other study that reports resistance value was 75% [27, 28, 29]. The improved occurrence of resistant to ceftazidime is associated to the high use of antibiotics with beta lactam like amoxicillin.

REFERENCES

[1] Iglewski B, Delden C. Emerg Infect Dis 1998; 4: 551–558.
[2] Zhu H, Bandara R, Conibear T, Thuruthyil S, Rice S, Kjelleberg S, Givskov M, Willcox MD. Invest Ophthalmol Vis Sci 2004; 45, 1897–1903.
[3] Van Delden C, Iglewski B. Emerg Infect Dis 1998; 4: 551-560.
[4] Fleiszig S, Wiener-kronish J, Miyazaki H, Valias V, Mostov K, Kanada D. Infect Immun 1997; 65: 579-586.
[5] Nikbin V., Aslani M., Sharafi Z, Hashemipour M, Shahcheraghi F, Ebrahimipour G. Iranian J Microbiol 2012;4(3):118-123
[6] Ali H. Al-Marzoqi and Zahraa M. Al Taee. J Nat Sci Res 2013; 3(2): 69-74.
[7] Fegan M, Francis P, Hayward AC, Davis GH, Furest JA. J Clin Microbiol 1990; 28: 1143-1146.
[8] Yetkin G, Otlu B, Cicek A, Kuzucu C, Durmaz R. Am J Infect Control 2006; 34: 188-192.
[9] Khan A, Cerniglia C. Appl Environ Microbiol 1994; 60: 3739-3745.
[10] Masuda N, Sakagawa E, Ohya S. Antimicrob Agents Chemother 1995; 39: 645-649.
[11] Carmen Chifiriuc , Sorin Dinu , Marcela Bucur , Carmen Iordache , Otilia Banu , Olguta Dracea and Veronica Lazar. Int J Mol Sci 2010; 11:5273-5291.
[12] Oluwole Adebayo Daini and Chinyere Gertrude Charles-Onyeaghala. Global Advanced Research Journal of Microbiology 2012; 1(4):052-056.
[13] Olukoya DK, Daini O, Alabi SA, Coker OA, Odugbemi T, Akinrimisi EO. Eur J Epidemiol 1988; 4:306-309.
[14] Vivyan E, Hedges RW, Datta N. J Gen Microbiol 1972; 70:443-452.
[15] Clinical and Laboratory Standards Institute. “Performance standards for antimicrobial disk susceptibility testing,” in Proceedings of the 17th International Supplement, vol. 21, Wayne, Ind, USA, M100-311, 2000.
[16] Sadikot RT. Blackwell TS, Christman JW, Prince AS. Am J Respir Crit Care Med 2005; 171, 1209–1223.
[17] Smith L, Rose B, Tingpej P, Zhu H, Conibear T, Manos J, Bye P, Elkins M, Willcox M, Bell S, Wainwright C, and Harbour C. J Med Microbiol 2006;55:1641–1644.
[18] Shen K, Sayeed S, Antalis P, Gladitz J, Ahmed A. Infec Immun 2002; 74: 5272–5283.
[19] Lee D, Urbach JM, Wu G, Liberati NT, Feinbaum RL. Genome Biol 2006; 7: R90.
[20] Mohd-Zain Z, Turner SL, Cerden˜o-Ta´rraga AM, Lilley AK, Inzana TJ. J Bacteriol 2004; 186: 8114–8122.
[21] Wolfgang MC, Kulasekara BR, Liang X, Boyd D, Wu K. Proc Natl Acad Sci U S A 2003; 100: 8484–8489.
[22] Wolska K, Szweda P. Pol J Microbiol 2009; 78: 255–260.
[23] Ramsey DM, Wozniak DJ. Mol Microbiol 2005; 56:309–322.
[24] Cotar AI, Dinu, S, Chifiriuc MC, Banu O, Iordache C, Larion C, Bucur M, Dracea O, Lazar V. Rom Biotehnol Lett 2008; 13:3765–3770.
[25] Korvick JA, Marsh JW, Starzl TE, Yu VL. Surgery 1991; 109: 62-68.
[26] Javiya VA, Ghatak SB, Patel KR, Patel JA. Indian J Pharmacol 2008; 40(5): 230-234.
[27] Anupurba S, Bhattacharjee A, Garg A, Sen MR. Indian J Dermatol 2006; 51:286-8.
[28] Siva GP, Nor AS, Ramelah M. Malaysian J Med Sci 2009; 16 (2): 27-32.
[29] Ali Hussein Al-Marzoqi, Hayder ShO, Hussein JH, Hassanein KI, Zahraa MA, and Sarah KY. Advances in Life Science and Technology 2013; 7: 7-14.