Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Antibiotic Susceptibility Pattern, Molecular Characterization of Virulence Genes among *Pseudomonas aeruginosa* Isolated from Burn Patients

Mustafa D. Younis[1]*, Omar F. Bahjat[1], Sirwan A. Rashid[2]

[1]Department of General Biology, College of Science, Cihan University-Erbil, Kurdistan Region, Iraq, [2]Department of Biomedical Sciences, College of Science, Cihan University-Erbil, Kurdistan Region, Iraq.

ABSTRACT

In this research, a total of 150 samples were obtained from burn and wound patients admitted to the West Erbil Emergence Hospital during period from September 2020 to January 2021. Through cultural, morphological features, biochemical testing, and Vitek 2 Compact Systems, 40 isolates of *P. aeruginosa* have been identified. *P. aeruginosa* produced various pigments, including blue/green and yellow/green. The isolates of *P. aeruginosa* were subjected to 14 different antibiotics. Imipenem was the most effective antimicrobial agents against all *P. aeruginosa* isolates, and most of isolates showed high resistance degree to ampicillin 100%, chloramphenicol 100%, amoxicillin-clavulanic acid 100%, cefotaxime 100%, and penicillin 100% while for aztreonam 32.5%, meropenem 42.5%, tobramycin 45%, gentamycin 45%, amikacin 45%, ciprofloxacin 62.5%, ceftazidime 67.5%, and tetracycline 80%. All *Pseudomonas aeruginosa* isolates were screened using multiplex polymerase chain reaction (PCR) to check for the presence of Pvda, LasB, Protease, exoA, exoT, exoU, and plch on its genomic DNA. The findings have shown that Pvda was 55%, LasB 75%, protease 65%, exoA 60%, exoT 75%, exoU 60%, and plch 55% of isolates harbored these genes as a virulence genes.

Keywords: *Pseudomonas aeruginosa*, burn patients, antibiotic resistance, virulence genes, and multiplex PCR

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative aerobic non-spore rod with remarkable capacity to survive and persist under many environmental circumstances.[1] In both hospitals and communities, *P. aeruginosa* is a common, opportunistic human pathogen.[2] Burning and wound infections are a challenge because they slow down the healing process, promote cicatrix, and can lead to bacteremia, sepsis (or organ failure) syndrome, however, organ from several systems cannot regulate homeostasis on its own and need immediate treatment.[3] The most severe pathogenic burn injuries are bacteria and fungi. Multiple species biofilms are formed on burning injuries in 48–72 h of the wound injury.[4] Organisms are acquired by the patient's own skin, digestive, and respiratory flora, as well as association with contaminated environments and health-care providers.[5] The human skin is considered the principal protective layer of the body's tissues and may contribute to damage and destruction of bacteria transmitted to the internal blood tissue, which is rich in proteins.[6] Isolation and laboratory diagnosis is used to diagnose *P. aeruginosa* infection. This aerobic bacterium is needed and thrives in the majority of laboratory culture media. On pseudomonas agar (selective media) and cetrimide agar, bacteria can be isolated, warmth, no spores, flagella morphology, positive, exercise catalase, lactose intolerance (positive oxidase reaction), fruit odor (grape flavor), and ability to grow at 42°C are used to detect bacteria.[7] *P. aeruginosa* is a ubiquitous microorganism that can quickly develop resistance to various antibiotics of broad spectrum.[2] Moreover, in recent years, resistance to a broad range of antibiotics by these microorganisms has made it difficult to treat infections caused and leads to higher death rates.[8] The development of soluble pyocyanin pigment, a water-soluble blue-green compound formed in large amounts, is one of *P. aeruginosa* characteristics. Pyocyanin acts as an antibiotic against a variety of bacteria and fungi.[9] *P. aeruginosa* has a variety of virulence factors in

Corresponding Author:
Mustafa D. Younis,
Department of General Biology, College of Science, Cihan University-Erbil, Kurdistan Region, Iraq.
E-mail: mustafa.thanoon@cihanuniversity.edu.iq

Received: May 5, 2021
Accepted: June 15, 2021
Published: June 30, 2021

DOI: 10.24086/cuesj.v5n1y2021.pp36-41

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host defenses and infection. These factors include hemolysin production, pyocyanin production, gelatinase, and biofilm formation, which act by enhancing the damage of tissue and helping bacteria to avoid the action of antibiotics.[10] Another virulence factor such as exotoxin A, exoenzyme S, elastase, and sialidase, which are powerfully controlled by cell-to-cell signals. Exotoxin1A (ETA) plays1a major role in the pathogenesis1of infections caused by1this organism as the primary virulence factor provided by most *P. aeruginosa* isolates. Such exotoxins may contribute to leukopenia, acidosis, and blood circulation, necrosis of the liver, pulmonary edema, bleeding, and kidney tubular necrosis[11] so that the aim of this study is screening of antimicrobial sensitivity profile of *P. aeruginosa* and detection of certain virulence genes through PCR technique.

MATERIALS AND METHODS

Patients and Samples Collection

One hundred and fifty samples were collected 1from patients admitted to West Erbil Emergency Hospital, during the period from September 2020 to January 2021. Following collection, each sample was cultured on different culture media and *P. aeruginosa* was identified by cultural characters, biochemical methods, and Vitek 2 Compact System.

Antibiotic Sensitivity Pattern

The isolates were examined for antibiotic sensitivity in accordance with the National Committee for Clinica1 Laboratory Guidelines 1and the Antimicrobial Susceptibility Testing Protocols by disc diffusion method on Mueller-Hinton agar.[12] Adjustment of the bacterial inoculates to the Clinical and Laboratory Standards Institute of 0.5 McFarland standards.[12] A sterile cotton swab was used to disperse the sample inoculum to Mueller-Hinton agar. The antimicrobial products tested, including: Imipenem (IPM), ceftazidime (CAZ), ampicillin (AM), aztreonam (ATM), chloramphenicol (C), amoxicillin-clavulanic acid (AMC), amikacin (AK), cefotaxime (CTX), gentamycin (CN), ciprofloxacin (CIP), tetracycline (TE), penicillin (P), meropenem (MEM), and tobramycin (TOB) were placed aseptically and incubated overnight. The zones of inhibition were interpreted and measured.[12]

Color Production by *P. aeruginosa* Isolates

All isolates had been inoculated on cetrimide agar, incubated for 18–24 h by streaking method at 37°C, and then, the pigment production was examined.[13]

DNA Extraction Protocol

Two hundred microliters of overnight growth were centrifuged for 30 s at 13,000 rpm, after that 1.5 ml was separated from supernatant in 2 ml microcentrifugal tube. The pellet has been dissolved in 200 µl TL buffer, then removed and fully mixed with 20 ul proteinase K solutions to achieve a uniform suspension. The sample has been incubated in the water bath at 56°C for 10 min until the cells have been completely lysed. Two hundred microliters of GB buffer applied to the specimens, then by vortexing mixed thickly for approximately 15 s up to a uniform mixture and then incubated for 10 min at 56°C. Then, 200 µl of absolute ethanol is added and pipetted or vortexes. The lysate transferred carefully without wetting the rim into the spin column reservoir for 1 min at 10,000 rpm, and the column 1centrifuged the collection tube then discharged containing the flow-through solution a new 2 ml tube has been placed with the GeNet Bio genomic DNA purification column. Five hundred microliters of GW1 buffer were added0 then centrifuged for 1 min at 10,000 rpm, the flow-through discarded and the purification column placed back into the collection tube, 500 µl of GW2 was added to the GeNet Bio genomic DNA purification column, centrifuged for 1 min at 10,000 rpm. Then after centrifuging the tube, remove the flow-through and reassemble the spin column with its collection tube, again, centrifuge at 12,000 rpm for 12 min to extract ethanol completely and check that the droplet is not attached at the bottom of the tube. Then, 1.5 ml of the spin column moved to a new tube to do the elution. Two hundred microliters of the elution buffer were added to the center of the GeNet Bio genomic DNA purification kit column membrane. The genomic DNA elution kept a side at room temperature for 1 min and centrifuged for 1 min at 10,0000 rpm. Then, the purified DNA was immediately removed and stored at −20°C for further applications (PrimePrep Genomic DNA extraction kit, GeNet Bio, Korea)

Protocol of PCR Technique

PCR conducted for all genes was performed in a 25 µl of reaction volume. Master Mix tube contains l2.5 µl, forward and reverse primers with 1 µl for each primer, DNA template 1 µl, and finally sterile (D. W) deionized water 9.5 µl.[14]

Detection of Pvda, LasB, Protease, exoA, exoT, exoU, and plch Virulence Genes in *P. aeruginosa*

Multiplex PCR also was used for the detection of *Pvda, LasB, Protease, exoA, exoT, exoU and plch* genes in *Pseudomonas aeruginosa* genome as shown in Table 1:

Protocol of Agarose Gel Electrophoresis

To perform gel electrophoresis, a method of Judelson[15] was followed with minor modifications. Adding 1.2 g agarose to 100 ml 1x TBE buffer was used as an agarose gel, the mixture melted for 1–2 min in the microwave oven or until it was apparent and fully dissolved. Left to cool at 50°C, 10 µl of primary safe dye was carefully added to the agarose solution then thoroughly mixed with a gentle stirring. The tray borders are sealed with the tape and inserted into the tray the right comb. Then, the agarose gradually poured in the tray and any bubbles were removed with a disposable tip, then kept away to the side at room temperature, the agarose solidified (15–30 min). The tape was removed from the tray and then the tray was placed in the electrophoresis tank. The tank was filled with more TBE buffers so that the ge1 is completely under buffer. The PCR product loaded into the wells (15 µl) with loading buffer. Depending on the size of the PCR sample, the first well (5 ul) (1 kb or 100 bp) was used. The gel runs for 50 min at 100 V. Finally, the UV transilluminator and gel photographed.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions for conventional and multiplex PCR[14]

Target gene	Primer sequences	Amplified segment (bp)	Initial denaturation	denaturation	Annealing	Extension	Final extension
Pvda	F-GACTCAGGCAAC	l28l	96°C	96°C	55°C	72°C	72°C
	TGCAAC		5 min	1 min	1 min	2 min	10 min
	R-TTCAGGTGCTGG						
	TACAGG						
LasB	F-GGAATGAACGAAGCG	300	96°C	96°C	55°C	72°C	72°C
	TTCTC		5 min	1 min	1 min	2 min	10 min
	R-GGTCCAGTAGTAGCG						
	GTTGG						
Protease	F- ATTTCGCCGACTCC	752	96°C	96°C	55°C	72°C	72°C
	CTGTA		5 min	1 min	1 min	2 min	10 min
	R-GAATAGACGCCGCTG						
	AAATC						
exoA	F-AACCAGCTCAGCCAC	207	96°C	96°C	55°C	72°C	72°C
	ATGTC		5 min	1 min	1 min	2 min	10 min
	R- GCTGGCCCATTCGCTCCAGCGCT						
exoT	F-AATCGCCGTCCAACTGCA TGCG	l52	96°C	96°C	55°C	72°C	72°C
	R-TGTTCGCCGAGGTAC		5 min	1 min	1 min	2 min	10 min
	TGCTC						
exoU	F-CCGTTGTGGTGCCGT	l34	96°C	96°C	55°C	72°C	72°C
	TGAAG		5 min	1 min	1 min	2 min	10 min
	R-CCAGATGTTCACCGA						
	CTCGC						
plch	F-GAAGCCATGGGCTAC	307	96°C	96°C	55°C	72°C	72°C
	TTCAA		5 min	1 min	1 min	2 min	10 min
	R-AGAGTGACGAGGAGC						
	GGTAG						

RESULTS AND DISCUSSION

Collection of *P. aeruginosa* Isolates

A series of confirming tests were conducted to verify that out of 150 bacterial isolates only 40 belong to species of *P. aeruginosa*. These smear preparations of bacterial cells were Gram-negative rods, non-spore forming, arranged in single or short chains. The colonies were thin, rough, or smooth on solid media with flat edges and high appearance, but some were mucoid in aspect. These isolates were found non-lactose ferment creating negative pale yellow colonies on MacConkey agar and on blood agar shows β-hemolytic colonies. Because of the production of the soluble pyocyanin and pyoverdin which are water soluble, the colonies were surrounded by bluish color on nutrient agar. The colonies pigments in selective media (Cetrimide agar) are more apparent yellow-green pigment. Biochemical tests confirmed *P. aeruginosa* burn contamination confines, biochemical testing was negative for indole, TSI, positive for oxidase and catalase, positive for citrate, positive for urease (slowly hydrolysis the urea), all *P. aeruginosa* 40 isolates had been also confirmed using Vitek 2's Compact System bacterium ID method.

Antimicrobial Sensitivity Screening Test for *P. aeruginosa*

Forty *P. aeruginosa* isolates were screened for their resistance to (14) widely used antibiotics including amikacin, amoxicillin-clavulanic acid, ampicillin, cefotaxime, penicillin, ciprofloxacin, chloramphenicol, gentamycin, imipenem, meropenem, tetracycline, ceftazidime, aztreonam, and tobramycin. The results of antibiotic resistance pattern for the bacteria1 isolates understudy are shown in Table 2.

Olayinka[16] reported that 20% of *P. aeruginosa* isolated from clinical samples obtained from the surgical units of Ahmadu Bello University teaching hospital in Nigeria were sensitive for imipenem which disagreed with our results, imipenem and meronem are ß- lactam antibiotics that they have broad-spectrum activity against both Gram-negative and Gram-positive bacteria.[17] All bacterial isolates displayed a low resistance and the majority of *Enterobacteriaceae* isolates

Table 2: Percentage of resistance bacterial isolates to different antibiotics

Antibiotics	Symbo1	Total no. of isolates	No. of resistant isolates	% of resistant
Amikacin	AK	40	18	45
Ampicillin	AM	40	40	100
Amoxicillin-clavulanic acid	AMC	40	40	100
Aztreonam	ATM	40	13	32.5
Chloramphenicol	C	40	40	100
Ceftazidime	CAZ	40	27	67.5
Ciprofloxacin	CIP	40	25	62.5
Gentamycin	CN	40	18	45
Cefotaxime	CTX	40	40	100
Imipenem	IMP	40	0	0
Meropenem	MEM	40	17	42.5
Penicillin	P	40	40	100
Tetracycline	TE	40	32	80
Tobramycin	TOB	40	18	45

showed no resistance. It might be because they are reserve medicines and they are used as the last option in our hospital environment for multidrug-resistant bacteria which agreed with our result. Ebrahimpour[18] reported that all *P. aeruginosa* isolated from burn patients were sensitive to IMP. This may be attributed to the inability of *P. aeruginosa* to produce enzymes that degrade or inactivate the antibiotic. Therefore, IMP is the most effective drug for the treatment of infections caused by *P. aeruginosa*. In the case of Fattma,[19] 98% of *P. aeruginosa* isolates resist amikacin, 96% for cefotaxime, 80% for rifampicin, 70% for ampicillin, 70% for augment, and 60% for doxycycline, which is near with our performance. Resistance by *P. aeruginosa* can both be due to inducible of beta-lactamases, which can make cephalosporin of broad-spectrum inactive and to beta-lactamases mediated by plasmid, which can lead to several penicillin's and ancient cephalosporin becoming resistant.[20] Mechanisms of aminoglycoside resistance in clinical isolates are usually controlled by enzymatic antibiotic inactivation since nine different enzymes that are capable of catalyzing phosphorylation, acetylation, and aminoglycosides coradenylylation in bacteria had been described.[21] The development of *P. aeruginosa* multiresistant and its antibiotics mechanisms involves decreased cell permeability, efflux pumps, and changes in target enzymes and antibiotics inactivation.[22]

Detection of Pvda, LasB, Protease, exoA, exoT, exoU, and plch Virulence Genes by Multiplex PCR in *P. aeruginosa*

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in l988,[23] this method has been successfully applied in many areas of DNA testing, including analyses of deletions,[24] mutations[25] and polymorphisms,[26] or quantitative assays[27] and reverse transcription PCR.[28] The role of various reagents in PCR has been discussed,[29] and protocols for multiplex PCR have been described by a number of groups. However, few studies[30] have presented an extensive discussion of some of the factors (e.g. primer concentration and cycling profile) that can influence the results of multiplex analysis. In the present study, 40 isolates of *P. aeruginosa* were tested for the detection of some virulence genes using polymerase chain reaction (multiplex). In our study, detection of virulence genes results showed that *Pvda* was 55%, *LasB* 75%, *Protease* 65%, *exoA* 60%, *exoT* 75%, *exoU* 60%, and *plch* 55% among tested strains, as shown in Figures 1 and 2.

Other findings also showed that in 100 strains of *P. aeruginosa,* all the virulence genes studied were detected. Therefore, the virulence genes studied might carry strains isolated from bovine meat, fresh fish, and smoked fish. The analysis revealed that the *LasB* genes are most frequently detected (89.0%) and *exoS* genes (84.0%) which could be explained by the fact that *P. aeruginosa*, secrets elastase (*LasB*).[31] The previous studies showed a high *LasB* prevalence in *P. aeruginosa* despite its isolated origin.[32] Another studies obtained by Holban[33] also agreed with our results, who reported that *lasB* 55%, *Protease* 75%, *exoT* 95%, *and plch* 55%, these virulence genes were detected using multiplex PCR, in *P. aeruginosa* which isolated from wound secretions. Mitov[34] also agrees with our results, who found that 100% for *lasB* and 71% for *plcH*, *the protease and lasB* both genes encode for proteases activity, and they are found in the majority of tested strains, lending support to the phenotypic data demonstrating that isolates obtained from burned patients can undergo hemolysis. *ExoS*, *exoT*, and *exoA* related exotoxins were distributed differently amongst the genes codified for the Type III Secretion System (T3SS). The most positive for T3SS exotoxins which encode genes in isolates from burn patients were also followed by tracheobronchial isolates. *ExoU,* codified for a major enzyme involved in pyoverdine synthesis, codifies for a highly cytotoxic exoenzyme *ExoU and PVdA* gene.[34]

P. aeruginosa has been estimated to be involved in between 10% and 22.5% of HAI both in adults and in children,[13] leading to increased costs for health care and prolonged hospital admission, respectively.[35] The clinical results of an infection with a combination of bacteria-related factors (intrinsic and

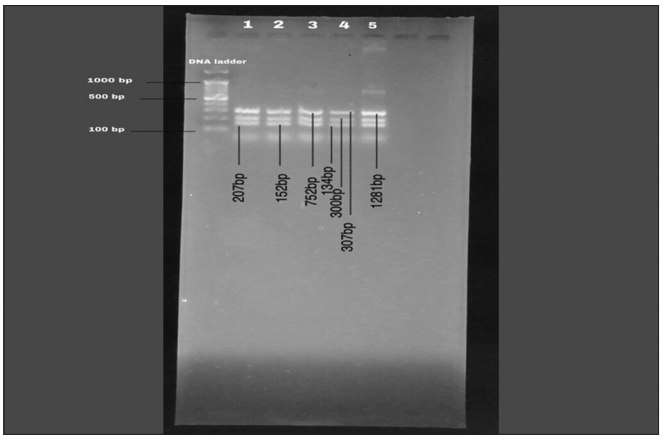

Figure 1: PCR amplification of *exoT, exoU, exoA, plch, Pvda, LasB, and Protease* virulence genes of *P. aeruginosa* through multiplex PCR, lanes (l, 2, 3, 4, and 5) represent amplified genes with product size amplicon size (152 bp, 134 bp, 207 bp, 307 bp, 1281 bp, 300 bp, and 752 bp) of *P. aeruginosa* isolates, M representing the ladder 100 bp

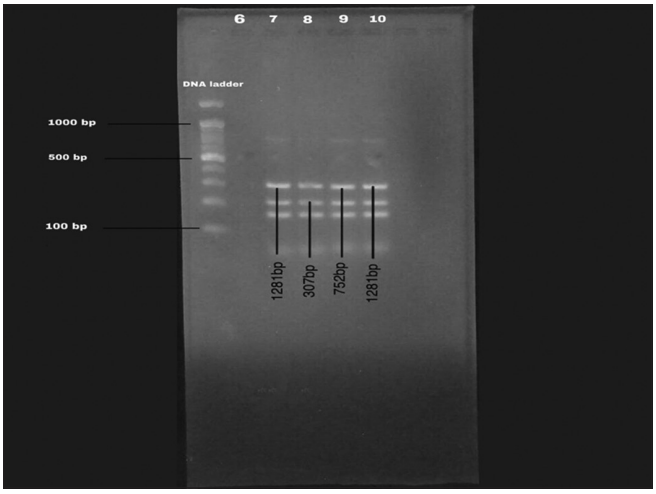

Figure 2: PCR amplification of *plch, Pvda, and Protease* virulence genes of *P. aeruginosa* through multiplex PCR, lanes 6 negative control, lanes 7, 8, 9, and 10 represent amplified genes with product size amplicon size (307 bp, 1281 bp, and 752 bp) of *P. aeruginosa* isolates, M representing the ladder 100 bp

antimicrobial resistance, prevalence and persistence in the hospital environment, and cocktail expression of a virulence) and individual differences in host susceptibility. In favorable environmental conditions, bacterial virulence is reduced and greatly increased if stressful conditions arise.[36]

CONCLUSION

P. aeruginosa showed resistance to most antibiotics, and imipenem was the most effective antibiotic against *P. aeruginosa* isolated from burn patients. Seven virulence genes were detected through the amplification of *Pvda, LasB, Protease, exoA, exoT, exoU*, and *plch* by multiplex PCR.

REFERENCES

1. S. Santajit and N. J. B. Indrawattana. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *BioMed Research International*, vol. 2016, p. 2475067, 2016.
2. K. Singh, M. Panghal, S. Kadyan and U. Chaudhary, J. P. J. Yadav. Antibacterial activity of synthesized silver nanoparticles from *Tinospora cordifolia* against multi drug resistant strains of *Pseudomonas aeruginosa* isolated from burn patients. *Journal of Nanomedicine and Nanotechnology*, vol. 5, no. 2, p. 1, 2014.
3. G. Héry-Arnaud, E. Nowak, J. Caillon, V. David, A. Dirou, K. Revert, M. R. Munck, I. Frachon, A. Haloun, D. Horeau-Langlard, J. Le Bihan, I. Danner-Boucher, S. Ramel, M. P. Pelletier, S. Rosec, S. Gouriou, E. Poulhazan, C. Payan, C. Férec, G. Rault, G. Le Gal and R. Le Berre. Evaluation of quantitative PCR for early diagnosis of *Pseudomonas aeruginosa* infection in cystic fibrosis: A prospective cohort study. *Clinical Microbiology and Infection*, vol. 23, no. 3, pp. 203-207, 2017.
4. S. Alkaabi. Bacterial isolates and their antibiograms of burn wound infections in burns specialist hospital in Baghdad. *Baghdad Science Journal*, vol. 10, no. 2, pp. 331-340, 2013.
5. M. E. Altaai, I. H. Aziz and A. A. Marhoon. Identification *Pseudomonas aeruginosa* by 16s rRNA gene for differentiation from other *Pseudomonas* species that isolated from patients and environment. *Baghdad Science Journal*, vol. 11, no. 2, pp. 1028-1034, 2014.
6. M. D. Y. Ali and Z. F. A. Abdulrahman. Molecular identification, susceptibility pattern, and detection of some virulence genes in *Pseudomonas aeruginosa* isolated from burn patients. *Plant Archives*, vol. 20, no. 1, pp. 2573-2580, 2020.
7. M. Hallin, A. Deplano, S. Roisin, V. Boyart, R. De Ryck, C. Nonhoff, B. Byl, Y. Glupczynski and O. Denis. Pseudo-outbreak of extremely drug-resistant *Pseudomonas aeruginosa* urinary tract infections due to contamination of an automated urine analyzer. *Journal of Clinical Microbiology*, vol. 50, no. 3, pp. 580-582, 2012.
8. R. J. Fair and Y. Tor. Antibiotics and bacterial resistance in the 21st century. *Perspectives in Medicinal Chemistry*, vol. 6, p. PMC. S14459, 2014.
9. H. A. Mohammed, H. S. Yossef and F. I. Mohammad. The cytotoxicity effect of pyocyanin on human hepatocellular carcinoma cell line (HepG2). *Iraqi Journal of Science*, vol. 55, no. 2B, pp. 668-674, 2014.
10. N. Cevahir, M. Demir, I. Kaleli, M. Gurbuz and S. Tikvesli. Evaluation of biofilm production, gelatinase activity, and mannose-resistant hemagglutination in *Acinetobacter baumannii* strains. *Journal of Microbiology, Immunology and Infection*, vol. 41, no. 6, pp. 513-518, 2008.
11. F. G. Tafesse, C. P. Guimaraes, T. Maruyama, J. E. Carette, S. Lory, T. R. Brummelkamp and H. L. Ploegh. GPR107, a G-protein-coupled receptor essential for intoxication by *Pseudomonas aeruginosa* exotoxin a, localizes to the Golgi and is cleaved by furin. *Journal of Biological Chemistry*, vol. 289, no. 35, pp. 24005-24018, 2014.
12. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing*. Wayne, PA: Clinical and Laboratory Standards Institute, 2011.
13. H. Loveday, J. Wilson, K. Kerr, R. Pitchers, J. T. Walker and J. Browne. Association between healthcare water systems and *Pseudomonas aeruginosa* infections: A rapid systematic review. *Journal of Hospital Infection*, vol. 86, no. 1, pp. 7-15, 2014.
14. A. Simonetti, E. Ottaiano, M. Diana, C. Onza and M. Triassi. Epidemiology of hospital-acquired infections in an adult intensive care unit: Results of a prospective cohort study. *Annali di Igiene*, vol. 25, no. 4, pp. 281-289, 2013.
15. H. Judelson. *Gel Electrophoresis. Protocol: Agarose Gel Electrophoresis Using Bio-Rad*, 2001.
16. A. Olayinka, B. Olayinka and B. A. Onile. Antibiotic susceptibility and plasmid pattern of *Pseudomonas aeruginosa* from the surgical unit of a university teaching hospital in North Central Nigeria. *International Journal of Medicine and Medical Sciences*, vol. 1, no. 3, pp. 79-83, 2009.

17. M. L. Joly-Guillou, M. Kempf, J. D. Cavallo, M. Chomarat, L. Dubreuil, J. Maugein, C. M. Serieys and M. Roussel-Delvallez. Comparative *in vitro* activity of Meropenem, Imipenem and Piperacillin/tazobactam against 1071 clinical isolates using 2 different methods: A French multicentre study. *BMC Infectious Diseases*, vol. 10, no. 1, pp. 1-9, 2010.
18. M. Ebrahimpour, I. Nikokar, Y. Ghasemi, H. S. Ebrahim-Saraie, A. Araghian, M. Farahbakhsh and F. Ghassabi. Antibiotic resistance and frequency of Class 1 integrons among *Pseudomonas aeruginosa* isolates obtained from wastewaters of a burn center in Northern Iran. *Annali di Igiene*, vol. 30, no. 2, pp. 112-119, 2018.
19. A. Fattma, M. Ali, Z. A. S. Asaad and M. Shkofa. Isolation and identification of multi drug resistant *Pseudomonas aeruginosa* causing wound infection in Erbil City. *International Journal of Research Studies in Science, Engineering and Technology*, no. 4, 2017, pp. 30-36.
20. J. G. Collee, T. J. Mackie and J. E. McCartney. *Mackie and McCartney Practical Medical Microbiology*. United States: Harcourt Health Sciences, 1996.
21. I. Aibinu, T. Nwanneka and T. Odugbemi. Occurrence of ESBL and MBL in clinical isolates of *Pseudomonas aeruginosa* from Lagos, Nigeria. *Journal of American Science*, vol. 3, no. 4, pp. 81-85, 2007.
22. P. A. Lambert. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *Journal of the Royal Society of Medicine*, vol. 95, no. Suppl 41, p. 22, 2002.
23. S. Verheij, J. Harteveld and T. Sijen. A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples. *Forensic Science International: Genetics*, vol. 6, no. 2, pp. 167-175, 2012.
24. C. S. Carlson, R. O. Emerson, A. M. Sherwood, C. Desmarais, M. W. Chung, J. M. Parsons, M. S. Steen, M. A. LaMadrid-Herrmannsfeldt, D. W. Williamson, R. J. Livingston, D. Wu, B. L. Wood, M. J. Rieder and H. Robins. Using synthetic templates to design an unbiased multiplex PCR assay. *Nature Communications*, vol. 4, no. 1, pp. 1-9, 2013.
25. Y. Achermann, M. Vogt, M. Leunig, J. Wust and A. Trampuz. Improved diagnosis of periprosthetic joint infection by multiplex PCR of sonication fluid from removed implants. *Journal of Clinical Microbiology*, vol. 48, no. 4, pp. 1208-1214, 2010.
26. B. Richards, J. Skoletsky, A. P. Shuber, R. Balfour, R. C. Stern, H. L. Dorkin, R. B. Parad, D. Witt and K. W. Klinger. Multiplex PCR amplification from the CFTR gene using DNA prepared from buccal brushes/swabs. *Human Molecular Genetics*, vol. 2, no. 2, pp. 159-163, 1993.
27. H. J. Monstein, Å. Östholm-Balkhed, M. Nilsson, M. Nilsson, K. Dornbusch and L. J. A. Nilsson. Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in *Enterobacteriaceae*. *APMIS*, vol. 115, no. 12, pp. 1400-1408, 2007.
28. P. Qin, W. Qu, J. Xu, D. Qiao, L. Yao, F. Xue and W. Chen. A sensitive multiplex PCR protocol for simultaneous detection of chicken, duck, and pork in beef samples. *Journal of Food Science and Technology*, vol. 56, no. 3, pp. 1266-1274, 2019.
29. L. Poirel, T. R. Walsh, V. Cuvillier and P. Nordmann. Multiplex PCR for detection of acquired carbapenemase genes. *Diagnostic Microbiology and Infectious Disease*, vol. 70, no. 1, pp. 119-123, 2011.
30. W. J. Mason, J. S. Blevins, K. Beenken, N. Wibowo, N. Ojha and M. S. Smeltzer. Multiplex PCR protocol for the diagnosis of staphylococcal infection. *Journal of Clinical Microbiology*, vol. 39, no. 9, pp. 3332-3338, 2001.
31. M. Khattab, M. Nour and N. M. ElSheshtawy. Genetic identification of *Pseudomonas aeruginosa* virulence genes among different isolates. *Journal of Microbial and Biochemical Technology*, vol. 7, no. 5, pp. 274-277, 2015.
32. K. Streeter and M. Katouli. *Pseudomonas aeruginosa*: A review of their pathogenesis and prevalence in clinical settings and the environment. *IEM*, vol. 2, no. 1, pp. 25-32, 2016.
33. A. M. Holban, M. Chifiriuc, A. I. Cotar and C. Bleotu. Virulence markers in *Pseudomonas aeruginosa* isolates from hospital acquired infections occurred in patients with underlying cardiovascular disease. vol. 18, no. 6, pp. 8843-8854, 2013.
34. I. Mitov, T. Strateva and B. Markova. Prevalence of virulence genes among bulgarian nosocomial and cystic fibrosis isolates of *Pseudomonas aeruginosa*. *The Brazilian Journal of Microbiology*, vol. 41, no. 3, pp. 588-595, 2010.
35. D. Jr. Hayes, S. E. West, M. J. Rock, Z. Li, M. L. Splaingard and P. Farrell. *Pseudomonas aeruginosa* in children with cystic fibrosis diagnosed through newborn screening: Assessment of clinic exposures and microbial genotypes. *Pediatric Pulmonology*, vol. 45, no. 7, pp. 708-716, 2010.
36. M. Ledizet, T. S. Murray, S. Puttagunta, M. D. Slade, V. J. Quagliarello and B. Kazmierczak. The ability of virulence factor expression by *Pseudomonas aeruginosa* to predict clinical disease in hospitalized patients. *PLoS One*, vol. 7, no. 11, p. e49578, 2012.