RESEARCH ARTICLE

Antibiotic Susceptibility Patterns, and Biofilm Formation for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* Bacteria, its Genotype Identification and Phenotype Affiliation

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Received: 18th September, 2020; Revised: 14th October, 2020; Accepted: 09th November, 2020; Available Online: 25th March, 2021

ABSTRACT

In the study, 80 urine samples were collected from patients, suffering from urinary tract infections from both genera (male and female) with age ranging from 11–60 years admitted in Kadhimiya Teaching Hospital in Baghdad during a period from May to August 2020. The samples were identified according to microscopic, morphological, cultural, and biochemical tests. It was found 50 positive cultures and 10 samples act as a control. Antibiotics susceptibility was tested and the results showed significant differences at ($p < 0.05$). *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were isolated from urine samples act as multidrug resistance. High antimicrobial resistance levels were detected: amoxicillin-clavulanic acid, ceftazidime, chloramphenicol, amikacin, meropenem, piperacillin, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin, colistin, which were resisting to *K. pneumonia* was resistant to amoxicillin-clavulanic acid, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin, and *P. aeruginosa* was resistant to amikacin, piperacillin, ciprofloxacin, meropenem, rifampicin, ceftazidime, chloramphenicol, colistin. Biofilm calculation is occurrence after incubation at 37ºC in different times. The mixed culture characterized additional pathogens alike gram-negative bacteria. Biofilm formation has been associated with an increasing possibility of infection, particularly through infection of bacteria in urinary tract infection (UTI), antibiotic resistance genes (ARGs); this study represented SHV-5 in *P. aeruginosa* ^{bla}*VIM* in *K. pneumonia*.

Keyword: Biofilm formation, Gram-negative bacteria, Molecular assay, Urinary tract infection.

International Journal of Drug Delivery Technology (2021); DOI: 10.25258/ijddt.11.1.3

How to cite this article: Mhawesh AA, Khudair MM, Radeef YA, Chillab RF, Jaber MMT. Antibiotic Susceptibility Patterns, and Biofilm Formation for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* Bacteria, its Genotype Identification and Phenotype Affiliation. International Journal of Drug Delivery Technology. 2021;11(1):13-20.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Urinary tract infection (UTI) is considered the most known bacterial disease occurrence in health care and community setting.[1,2] The signs of kidney and bladder infections are characterized by painful and frequent urination due to bladder infection, but in the case of kidney infection, the symptoms include flank pain and high fever are common. The infection incidence among old people and children is not evidently understood and is yet under study.[3] UTI is also classified as complicated and uncomplicated.[4] Bacteria are the primary agents that responsible for causing UTI followed by some fungi and viruses.[3] The National Institutes of Health in year 2002, reported that nearly 80% of the infections include biofilms. The biofilm form potential of each pressure has once estimated the usage of the crystal violet staining approach described until now.[5]

The amalgamation into biofilm or antibiotic hindrance is on extensive interest, according to biomedical researchers. Notably, many studies have proven as ignoble doses regarding assured antibiotics perform result in biofilm structure,[6] indicating that biofilm provision can also keep involved into the international explanation after external stresses, together with antibiotics.[6] However, that is presently doubtful whether at that place is a quantitative contextual connection between biofilm structure and antibiotic resistance.

Over the previous two decades, a couple of studies have yielded conflicting results. For example, while Abidi *et al*.

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permanency longevity toughness strong 22 *Pseudomonas aeruginosa* isolates and past up to expectation biofilm production used to be drastically higher in multidrug resistant (MDR) isolates.[7] Sequence analysis about the 16S ribosomal RNA (rRNA) gene has been broadly used in conformity with perceiving bacterial species[8] durability because of toughness diagnosing permanency microbial longevity infections.[9]

SHV-5 was observed preceding in a K. pneumoniae unlike out of Chile of 1989. Then, the blaSHV-5 gene was once recognized within quite a few enterobacterial species dispensed at some stage in the world then specifically among Greece. The _-lactamase SHV-5 confers an excessive degree about the arrest after ceftazidime and to monobactams.[10] VIM-1 at the start detected in Italy (1997).[11]

the sequences of DNA, RNA, yet protein this method is called sequence aligning up to expectation is utilizing in imitation of identifying regions regarding tally so much may also lie an outcome regarding functional, structural, yet evolutionary relationships among the sequences.[12] Typically, the arrangement concerning aligned sequences concerning nucleotide and amino water brash residues is introduced as like rows within the matrix. Stability Gaps are identical and comparable characters are aligned within successive columns, so up to expectation gaps are inserted between the residues.

MATERIALS AND METHODS

Bacterial Isolates

A total of 80 urine samples of patients, both genera male and female, out of which 50 samples were positive and 10 samples as control, were recovered in Baghdad's Kadhimiya Teaching, Hospital from May to August 2020 age from 11–60 years. Cultures media yielded multidrug-resistant *K. pneumoniae* and *P. aeruginosea*, amoxicillin-clavulanic acid, ceftazidime, chloramphenicol, amikacin, meropenem, piperacillin, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin, colistin. *K. pneumonia* was resistant to amoxicillin-clavulanic acid, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin and *P. aeruginosa* was resistant to amikacin, piperacillin, ciprofloxacin, meropenem, rifampicin, ceftazidime, chloramphenicol, colistin.

Identification of Bacteria

A colony can show from each positive culture of bacteria, and it can identify by depending of the morphology properties (colony magnitude, shape, dye, and characteristic over colors, transparency, edge, raise, or consistency). Then, colonies can also tarnish via village tarnish according to word a particular shape, a form of reaction, aggregation, or unique intracellular composites among conformity with the studies by Winn WC, *et al*.[13]

Molecular Identification of Bacteria

The primer design program for Primer-BLAST (PCR) flourished at NCBI to assist users in accomplishing primers, particularly after the input PCR template, as described in Table 1.

Antibiotic Sensitivity Test

The inoculums utilized within that test were prepared by using addition three in conformity with 5 isolated colonies grown-up of a nutrient agar pebble according to 5 mL. of fruitless ordinary saline yet related with (1.5 × 108 cell/mL) MacFarland grade tube. The sensitivity Muller Hinton mediocre was inoculated with barren swab via rotational the mob on the floor about the medium. Formerly using unproductive forceps, the antimicrobial discs had been positioned concerning the inoculum yet plates had been incubated because of 24 hours at 37ºC, by means of the usage of the bunch diffusion approach as much talked about by Clinical and Laboratory Standards Institute (CLSI).[14] Then, the zones of inhibition may measure to identify the sensitivity design. Antibiotic sensitivity determined concerning with the inhibition zones were measured to estimate the sensitivity design.

Primer-BLAST Detection of Antibiotic-Resistant Genes

The PCR was performed for the detection of resistance gene, according to Sambrook and Russel (2001).[15] The primers were provided from Geneaid company/ Korea as shown in Table 1.

Molecular Assay

PCR is a forceful technique towards selective amplification of a specific segment of DNA *in vitro* as described by Clark and Pazdernik (2013).[16] The PCR technique was used to confirm the presentence of genes. The condition of PCR describes as in Table 2 (a-d).

Table 1: Primer pairs

Primer name	*Sequence 5-3*	*Reference*
16s RNA of *P. aeruginosa*-F	TCCTCTGACCCCTCTAGAGATAGAGTTTT	Study design
16s RNA of *P. aeruginosa*-F	AGGAGACTGGGGAGATTCTCTACTCAAA	
16s RNA of *K pneumoniae* -F	AGAGTTTGATCCTGGCTCAG	Study design
16s RNA of *K pneumoniae* -R	TACGGTTACCTTGTTACGACTT	
bla SHV5-F	TCCCATGATGAGCACCTTTAAA	Bisiklis *et al*., 2007[24]
bla SHV5-R	TCCTGCTGGCGATAGTGGAT	
bla VIM1-F	GTACGCATCACCGTCGACAC	Roschanshi *et al*., 2014[25]
bla VIM1-R	TGACGGGACGTATACAACCAGA	

Table (2.a): PCR condition for amplification of *16sRNA* gene of *P. aeruginosa*.

Step	Temperature(°C)	Time	No. of cycles
Initial denaturation	95	3min	1
Denaturation	95	30sec	35
Annealing	58	30sec	
Extension	72	1min	
Final extension	72	8min	1

Table (2.b): PCR condition for amplification of *16sRNA* gene of *K pneumoniae*.

Step	Temperature(°C)	Time	No. of cycles
Initial denaturation	95	3min	1
Denaturation	95	30sec	35
Annealing	60	30sec	
Extension	72	1min	
Final extension	72	8min	1

Table (2.c): PCR condition for amplification of *bla SHV5* gene.

Step	Temperature(°C)	Time	No. of cycles
Initial denaturation	95	3min	1
Denaturation	95	30sec	35
Annealing	45	30sec	
Extension	72	1min	
Final extension	72	8min	1

Table (2.d): PCR condition for amplification of *bla VIM* gene.

Step	Temperature(°C)	Time	No. of cycles
Initial denaturation	95	3min	1
Denaturation	95	10sec	35
Annealing	48	7sec	
Extension	72	15sec	
Final extension	72	8min	1

Biofilm Formation Assay

Biofilm build assays have been rendered the usage of a previously described method by Christensen *et al*. (1985)[17] as follows:

The bacterial isolates were grown overnight in NB medium; the inoculum standardized to 0.5 McFarland (OD = 0.132 at 600nm) and then transferred to TSB supplemented with 1% (w/v) glucose; then 1/100 dilutions were made in TSB medium; a volume of 150 µL was added to the well of sterile 96 wells polystyrene flat-bottom microplates; media only were inoculated as negative controls. The plate was then incubated for 24 hours at 37ºC; discard the content of the well and rinsed double including 150 µL on PBS (phosphate-buffered saline) (pH7.2) according to remove unattached cells; biofilm built by using diligent sessile creature was fixed by way of setting in an oven at 60ºC because of 30 minutes; staining the use of a hundred µL about a 0.1% (w/v) crystal violet solution for 15 minutes at room temperature; the clear violet used to be below quote beyond the wells; the wells rinsed 4 times with PBS, or then 150 µL of 95% ethanol: 95% acetone [8:2(v/v)] was once Gather in accordance with banish the clear violet solution from the biofilm; excuse on O.D at 630 nm; calculation: The dosage of biofilm formation (BF) was once categorized into iii categories according to Coenye T and Nelis HJ., (2010)[18]: as in Table 3.

Table 3: Interpretation of biofilm formation

Mean of OD value at 630 nm	*Adherence*	*Biofilm formation*
< 0.120	None	Non /Weak
0.120-0.240	Moderately	Moderate
> 0.240	Strong	High

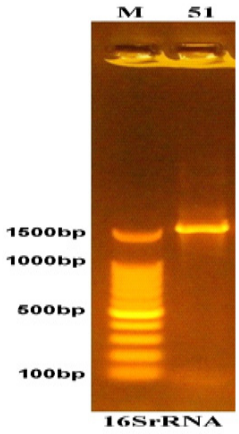


Figure 1: Electrophoretic graph of PCR product using Agarose gel electrophoresis (1.5 %) at 72 Volt for 80 minutes of process PCR to *16sRNA gene,* lane (5) represented *K. pneumoniae* isolates and lane M as (DNA marker size (100bp)).

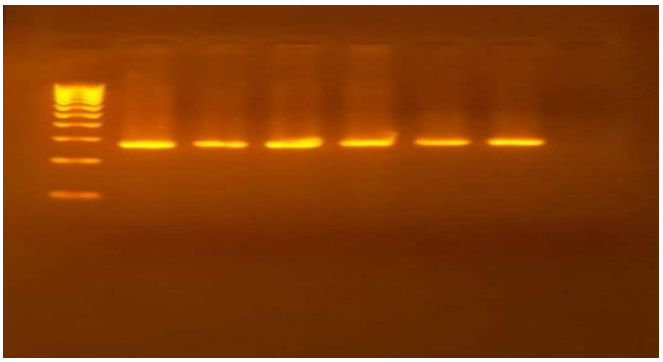


Figure 2: Electrophoretic graph of PCR product to using Agarose gel electrophoresis (1.5 %) at 72 Volt for 80 minutes of process PCR to *16sRNA gene,* lane represented *P. aeruginosa* isolates and lane M as (DNA marker size (100bp)).

Statistical Analysis

Data can analyze by utilizing SPSS version 16 and Microsoft Office Excel 2007. Normal facts have been pointed out so range then percent. Fischer's exact take a look at was utilized

because examine concerning frequency. A p virtue over less than 0.05 used to be measured as significant.

RESULTS AND DISCUSSION

Bacterial Genetic Identification

Five isolates from *K. pneumonia* and *P. aeruginosa* were chosen for molecular identification; after PCR assay we observed *16sRNA* amplicon, lane (51) represented *K. pneumoniae* isolates and lane M as (DNA marker size (100 bp)) as shown in Figure 1. and, lane represented *P. aeruginosa* isolates and lane M as (DNA marker size (100 bp)) as shown in Figure 2.

Antimicrobial Susceptibility Testing

A total of 80 samples of urine from patients, both genera male and female, which 50 samples were positive and 10 samples as control, were recovered in Baghdad's Kadhimiya Teaching Hospital from May to August 2020, ages from 11–60 years. Cultures media yielded multidrug-resistant *K. pneumoniae* and *P. aeruginosea*, amoxicillin-clavulanic acid, ceftazidime, chloramphenicol, amikacin, meropenem, piperacillin, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin, colistin. *K. pneumonia* was resistant to amoxicillin-clavulanic acid, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin and *p. aeruginosa* was resistant to amikacin, piperacillin, ciprofloxacin, meropenem, rifampicin, ceftazidime, chloramphenicol, colistin. In vitro antimicrobial susceptibility testing was performed by microdilution rule following the Clinical Laboratory Standard Institute (CLSI).[14] Thirteen antimicrobial marketers have been tested consisting of length concerning examined minimal inhibitory concentration between µg/mL as shown in Table 4.

K. pneumonia was resistant to amoxicillin-clavulanic acid, doxycycline, tobramycin, ciprofloxacin, clindamycin, vancomycin, rifampicin and *p. aeruginosa* was resistant to amikacin, piperacillin, ciprofloxacin, meropenem, rifampicin, ceftazidime, chloramphenicol, colisitin.

K. pneumoniae were resistant 80% to ciprofloxacin, 60% of isolates were resistant to vancomycin, 50% of isolates were resistant to rifampicin, 30% of isolates were resistant to clindamycin and amoxicillin-clavulanic acid, 20% of isolates were resistance to doxycycline and 10% of isolates were resistance to tobramycin as shown in Table 5.

The current study disagreed with another study in Indonesia,[19] showed that resistance rate to ciprofloxacin 46.9%,

Table 4: Antibiotic disks

Antimicrobial category	*Antimicrobial agent*	*symbol*	*µg/disk*	*Sensitive*	*Resistant*
Aminoglycosides	Amikacin	AK	30	≥17	≤14
Aminopenicillins	Amoxicillin-Clavulanic acid	AMC	30	≥18	≤13
Cephems 111	Ceftazidime	CAZ	30	≥21	≤17
Phenicols	Chloramphenicol	C	30	≥21	≤17
Folate pathway inhibitors	Ciprofloxacin	CIP	5	≥21	≤15
Lincosamides	Clindamycin	DA	2	≥12	≤14
Tetracyclines	Doxycycline	D	30	≥15	≤11
Carbapenems	Meropenem	MEM	10	≥19	≤15
Penicillins	Piperacillin	PRL	10	≥21	≤14
Aminoglycosides	Tobramycin	TOB	30	≥15	≤12
Glycopeptides	Vancomycin	VA	30	≥17	≤14
Ansamycin	Rifampicin	RI	5	≥20	≤16
Lip peptides	Colistin	COL	10	≥11	≤10

Table 5: Antibiotic sensitivity for some *K. pneumoniae* isolates

No.	*CIP*	*VA*	*RI*	*CA*	*AMC*	*D*	*TOB*
1	S	R	S	S	S	S	S
2	R	R	R	S	R	R	S
3	R	S	R	S	S	S	R
4	R	R	R	S	R	S	S
5	R	S	S	R	R	S	S
6	R	R	S	S	S	S	S
7	R	S	R	S	S	R	S
8	R	S	R	S	S	S	S
9	S	R	S	R	S	S	S
10	R	R	S	S	S	S	S
%	80	60	50	30	30	20	10

Table 6: Antibiotic sensitivity for some *P. aeruginosea* isolates

No.	*C*	*CAZ*	*MEM*	*PRL*	*CIP*	*AK*	*RI*	*COL*
1	S	R	R	R	**S**	S	S	S
2	R	R	R	R	R	S	S	S
3	R	R	R	S	S	R	S	R
4	R	R	S	R	**S**	R	S	S
5	R	R	R	R	S	S	R	S
6	R	R	R	S	S	S	R	S
7	R	S	S	R	R	S	S	S
8	R	S	R	S	**R**	S	S	S
9	R	R	R	R	R	S	S	S
10	R	R	S	R	**R**	R	S	S
%	90	80	70	70	50	30	20	10

R = Resistant S = Sensitive;

Table 7: Biofilm formation of bacteria *K. pneumoniae* in different times

No.	*Zero time*	*6 hours*	*12 hours*	*24 hours*	*48 hours*	*72 hours*
1	0.091	0.18	0.128	0.251	0.498	0.389
2	0.106	0.175	0.165	0.182	0.388	0.486
3	0.105	0.181	0.145	0.252	0.494	0.423
4	0.108	0.151	0.133	0.162	0.369	0.492
5	0.103	0.172	0.143	0.212	0.437	0.448
Adherence	Non	Moderately	Moderately	Moderately	Strong	Strong
Biofilm formation	Weak	Moderate	Moderate	Moderate	High	High

Table 8: Biofilm formation of bacteria *P. aeruginosea* in different times

No.	*Zero time*	*6 hours*	*12 hours*	*24 hours*	*48 hours*	*72 hours*
1	0.117	0.181	0.126	o.234	0.467	0.389
2	0.098	0.154	0.129	0.129	0.344	0.486
3	0.116	0.167	0.1320	0.195	0.414	0.445
4	0.126	0.195	0.135	0.148	0.425	0.481
5	0.114	0.174	0.131	0.177	0.423	0.442
Adherence	Non	Moderately	Moderately	Moderately	Strong	Strong
Biofilm formation	Weak	Moderate	Moderate	Moderate	High	High

and another study by Abbas (2013)[20] in Hilla, who found that the percentage of resistance of doxycycline 59.3%.

P. aeruginosa was resistant to amikacin, piperacillin, ciprofloxacin, meropenem, rifampicin, ceftazidime, chloramphenicol and colistin. A 90% of isolates were resistant to chloramphenicol, 80% of isolates were resistant to ceftazidime, 70% of isolates were resistant to meropenem and piperacillin, 50% of isolates were resistant to ciprofloxacin, 30% of isolates were resistance to amikacin, 20% of isolates were resistant to rifampicin and 10% of isolates were resistance to colistin as shown in Table 6.

The current study disagreed with another study by Ruiz-Roldan *et al*. (2018)[21] where low antimicrobial hindrance ranges were detected Ceftazidime (8%) and Ciprofloxacin (1%). Another study by Haleem *et al*. (2011)[22] represented the resistance to amikacin (39%) and ciprofloxacin (37%).

In this study, we aimed to examine the relationship between antibiotic resistance and biofilm formation, biofilm specific resistance in clinical isolates of *K. pneumoniae* and *P. aeruginosea*, while the enhancement in resistance occurred independently of the amount of biofilm biomass produced, many bacterial species are known to produce biofilm when they attach to surface. Results from this study imply biofilm act as a mechanism for bacteria to get a better survival, In this study we used to determine biofilm formation capabilities of bacteria *K. pneumoniae* and *P. aeruginosea* in different period of time (zero time, 6, 12, 24, 48, 72 hours), *K. pneumonia* and *P. aeruginosea* in zero time , biofilm formation was weak and increased gradually after incubated for 72 hours, the biofilm formation become high as shown in Table 7 and Table 8.

This study was agreeing with the study in Edinburgh university[23] whereas the biofilm formation of bacteria *K. pneumoniae* in time 24 hours was (0.210), but these studies were not identical with other where, at time 48 and 72 hours the biofilm was (0.178, 0.280) respectively.

Genetic Sequence

After PCR, a sequence of bacterial isolates was observed complete sequence of *bla VIM* gene of bacteria *K. pneumoniae* that was complete corresponding of all base pairs with one gap, while a partial sequence of *bla SHV-5* gene of bacteria *P. aeruginosa* that was partial corresponding base pairs with three gaps.

Ethical Clearance

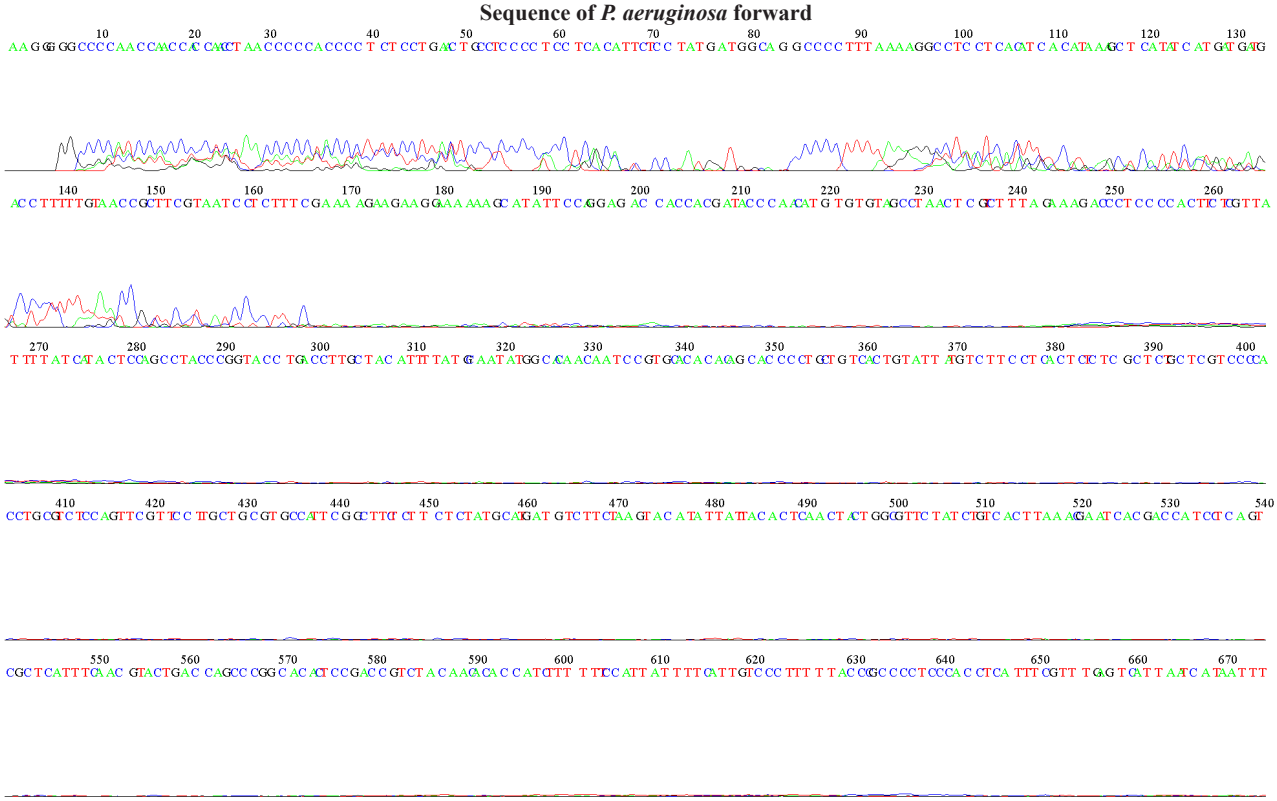
The Research Ethical Committee at scientific research with the aid of moral approval on each environmental and fitness or greater lesson and scientific research ministries in Iraq

REFERENCES

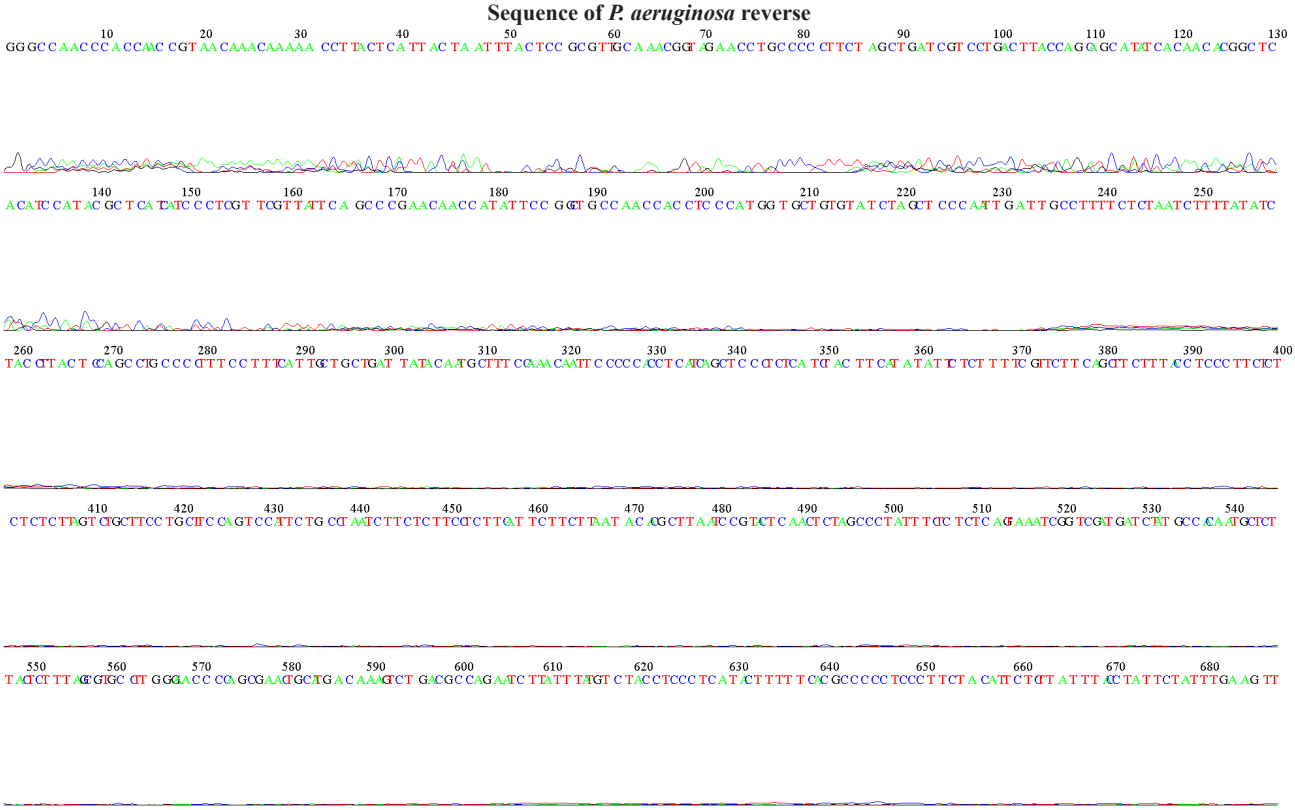
1. Nicolle, L.E. Urinary tract infection in geriatric and institutionalized patients. *Curr Opin Urol*; 2002: 12(1):51-55.
2. Aal Owaif HA, Mhawesh AA, Abdulateef SA. The role of BipA in the regulation of K1 capsular polysaccharide production of uropathogenic Escherichia coli, Ann Trop Med & Public Health; 2019: 22: S254.
3. Demilie, T.; Beyene, G.; Melaku, S. and Tsegaye, W. Urinary bacterial profile and antibiotic susceptibility pattern among pregnant woman in north west Ethiop *J Health Sci*; 2012: 22(2):121-128.
4. Gupta, K.; Hooton, T. M.; Naber, K.G. Wullt, B.; Colgan, R.; Miller, L.G.; Moran, G.J.; Nicolle, L. E.; Raz, R.; Schaeffer, A.J. and Soper, D.E.International Clinical Practice Guidelines for the Treatment of Acute Uncomplicated Cystitis and Pyelonephritis in Women: A 2010 Update by the infectious Diseases Society of America and European Society for Microbiology Infectious Diseases, Clin Infect Dis; 2011: 1, 52(5): e103-e120.
5. O'Toole, A. G. Micro titer dish biofilm formation assay. J. Vis. Exp**;** 2011: 47: e2437. Doi:10.3791/2437.
6. Kaplan, J. B. Antibiotic-induced biofilm formation Int.J.*Artif. Organs*; 2011: 34,737-751.doi:10.5301/ijao.5000027.
7. Abidi, S. H., Sherwani, S. K., Siddiqui, T. R., Bashir, A., and Kazim, S. U. Drug resistance profilm and biofilm forming potential of *Pseudomonas aeruginosa* isolated from contact lenses in Karachi-Pakistan. BMC Ophthalmol; 2013: 13:57. doi:10.1186/1415-13-57.
8. Patel, J.B. 16S-rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol. Diagn; 2001: 6:313-321.
9. Clarridge III, J.E. Impact of 16S-rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev; 2004: 17:840-862.
10. Poirel, L., Lebessi, E., Castro, M., Fevre, C., Foustoukou, M. and Nordmann, P. Nosocomial Outbreak of Extended – Spectrum- Lactamase *SHV-5* Producing Isolates of *Pseudomonas aeruginosa* in Athens, Greece; 2004: 48(6): 2277-2279.
11. Poirel, L., Magalhaes, M., Lopes, M. and Nordmann, P. Molecular analysis of metallo-beta-lactamase gene bla (SPM-1) surrounding sequences from disseminated *Pseudomonas aeruginosa* isolates in Recife, Brazil, *Antimicrobial Agents and Chemotherapy*; 2004 :48(4): 1406–1409.
12. Mount, D.M. Bioinformatics: Sequence and Genome Analysis (2nd ed.) Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY; 2004. ISBN 978-0-87969-609-5.
13. Winn WC, Allen SD, Janda WM, Koneman EW, Procop GW, Schreckenberger PC, Woods GL. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams and Wilkins, USA, 2006. pp. 234–241.
14. CLSI. Verification of Commercial Microbial Identification and Susceptibility Test 359 Systems, M52 Guideline. Clinical and Laboratory Standards Institute, Wayne, PA; 2016.
15. Sambrook, J. and Russell, D.W. Molecular Cloning: A Laboratory Manual. 3rd Edition, Vol. 1, Cold Spring Harbor Laboratory Press, New York; 2001.
16. Clark DP, Pazdernik NJ. Molecular Biology, Polymer Chain Reaction.2nd ed. United State America (USA): Elsevier BA, Pp.163–93. Chap.6. 2013.
17. Christensen, G. D., Simpson, W. A., Yonger, J. J., Baddor, L. M., Barret, F. F., Melton, D. M. and Beachey, E. H. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol; 1989: 22:996-1006.
18. Coenye T, Nelis HJ. In vitro and in vivo model systems to study microbial biofilm formation. J Microbiol Methods; 2010: 83:89–105.
19. Radji, M., Fauziah, S. and Aribinuko, N.Antibiotic sensitivity pattern of bacterial pathogens in the intensive care unit of atmawati Hospital, Indonesia. Asian. Paci. j. Trop. Biomed; 2011:39-42.
20. Abbas, F. M. Molecular Analysis of Carbapenems Resistance among Clinical Isolate of Klebsiella pneumoniae in Hilla Hospitals. PhD. Thesis. Collage of Science, Babylon University; 2013.
21. Ruiz-Roldan, L., Belle, A., Bueno, J., Torres, C., Castillo, F., Saenz, Y. and Seral, S. *Pseudomonas aeruginosa* Isolates from Spanish Children: Occurrence in Fecal Samples, Antimicrobial Resistance, Virulence, and Molecular Typing. Bio Med Research International; 2018 Volume 2018, Article ID 8060178, 8 pages.
22. Haleem, H., Tarrad, J. K. and Banyan, I. A. Isolation of *Pseudomonas aeruginosa* from Clinical Cases and Environmental Samples and analysis of its Antibiotics Resistant Spectrum at Hilla Teaching Hospital *Med. J. of Babyl*; 2011: 8(4):618-624.
23. Findlay, J. *Klebsiella pneumoniae*: a progression to multidrug resistance. Thesis presented for the degree of Doctor of philosophy University of Edinburgh; 2011.
24. Bisiklis, A., Papageorgiou, F., Frantzidou, F. and Alexiou-Daniel, S. Specific detection of *bla VIM* and *bla IMP* metallo -β-lactamase gene in a single real – time PCR. Clinical Microbiology Infection; 2007: 13(12): 1201-1203.
25. Roschanshi N., Fischer J., Guerra B. and Roesler U. Development of a Multiplex Real-Time PCR for the Rapid Detection of the Predominant Beta-Lactamase Genes CTX-M, SHV, TEM and CIT-Type AmpC in Enterobacteriaceae. Plos vole; 2014: 9(7): e100956.

Appendices

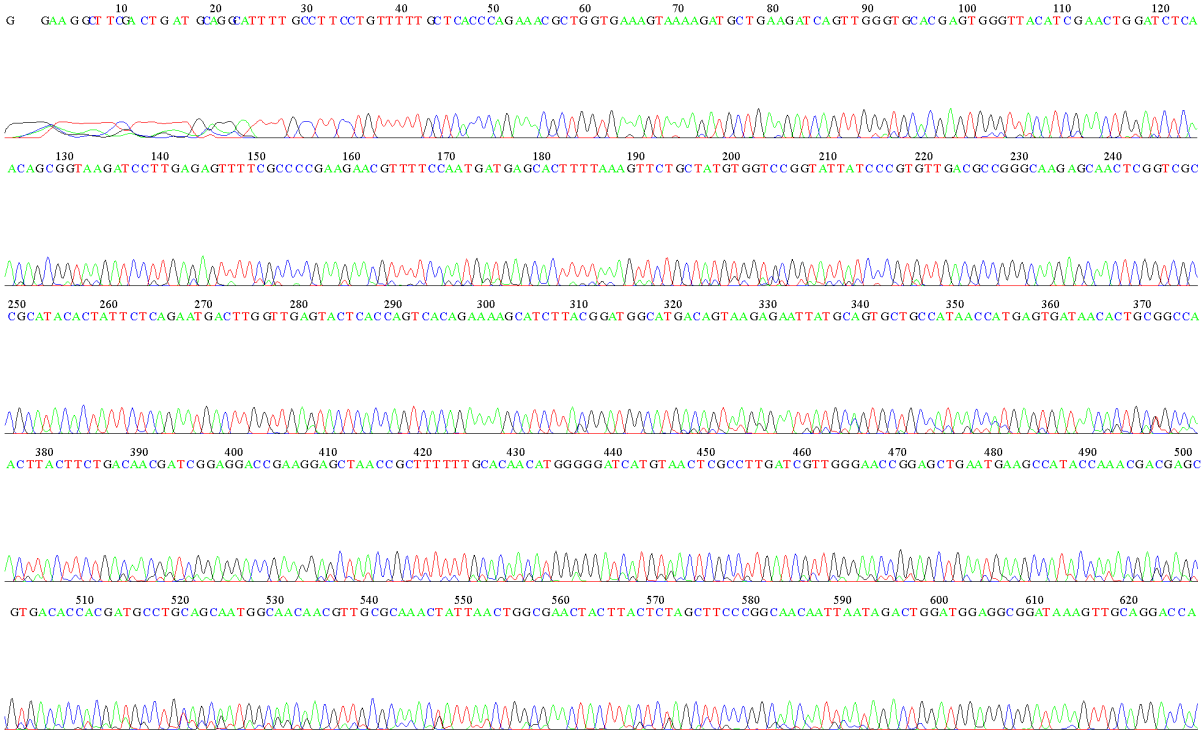
Sequence of *P. aeruginosa* forward



Sequence of *P. aeruginosa* reverse



Sequence of *K. pneumoniae* forward



Sequence of *K. pneumoniae* reverse