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DETECTION OF *CASP-5 GENE* AS INFLAMMATORY FACTOR IN IRAQI PATIENTS WITH *PSEUDOMONAS AERUGINOSA* INFECTIONS

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ABSTRACT

Pseudomonas aeruginosa is define as one of an aerobic Gram-negative bacteria that considered as one of most problematic nosocomial emerged pathogens. This study was performed on 50 isolates and blood patients. Definitive diagnosis of positive P. *aeruginosa* isolates based on the characteristic examination, the phenotypic of colonies and microscopic morphology which grown in media

also biochemical tests for diagnosis of isolates Fifty isolates identified as *Pseudomonas aeruginosa*, the percentage of isolates according to clinical sources were 19(38%) from burn, 12 (24%) from urine,3(6%) from ear swabs,10(20%) from sputum and 6(12%) from wounds. The diagnosis was conducted and confirmed by using API20E the result of it is 47(94%). The results showed isolates a different ability to resist antibiotics. Amoxicillin- clavulanic acid, Trimethoprim-sulfamethoxazole and Erythromycin were the effectiveness antibiotic against *P. aeruginosa* 100%.The ability of biofilm formation as a virulence factor, Micro-titer plate method (MTP) was used, results revealed that (46) isolates (92%) of *P. aeruginosa* have the ability to form biofilm by MTP method. Genotypic detection was done by conventional PCR for *16SrDNA* gene by using specific primer (*pa-ss*) 50(100%) to detect the virulence gene (*Flic* gene) which is virulence factor gene in *p. aeruginosa* responsible for activation of caspase-1enzyme, the result showed 45(90%) positive. all the blood sample the results were positive %100 . Sequencing for 20 casp-5 PCR product to detect variations in gene of Iraqi population and the results showed 20 sample from 20 have a genetic variation, heterozygous nucleotide.

Keywords: *Pseudomonas aeruginosa,* Flagillin, *flic* and *Casp-5 gene*.

INTRODUCTION

The P. aeruginosa is emerging pathogens that considered common types of problematic nosocomial causes infection in persons of low or depressed immunity as opportunistic pathogen (Brooks *et al.,* 2007). It is the infectious leading causes in wound, urinary tract, ear and surgical wound (Todar, 2008) The system of *Pseudomonas* detection are very important of patients diagnosis and prevention further spreading of the infection. The most important techniques that still used in microbiological diagnosis is Cultivation of bacteria due to its capability to detection viable bacteria quantity in a sample, in addition to purified sample for another testing (Minion, 2010) For identification, a lot of studies satisfied by using the classical biochemical test or API 20E system (Capuzzo *et al.,* 2005) however, the sensitivity of these methods are effected by *adaptive* ability of *P. aeruginosa* that causes a difficulties. Therefore, it mandatory to develop genotype-based characterization systems that able accurately for identifying these micro-organisms despite any phenotypic modifications, DNA marker allow the species identification rapidly. The highly sensitive specific and rapid method among DNA markers is the Polymerase Chain Reaction (PCR) that improved the *P. aeruginosa* detection (Xu *et al.,* 2005) to diagnosis differentiate *Pseudomonas* species in environmental and clinical samples specific *16S rRNA* gene was used (Spilker *et al.,* 2004). Bacterium is motile by single polar flagellum for swimming motility, spreading chemotaxis, in the environment, ability to transferring to preferred host, colonization sites optimize, and ewsponse to the Toll-like receptor 5 (TLR5)-dependent inflammatory (McIsaac *et al.,* 2012) the flagellum contributing in bacterial pathogenicity as virulence factor (Haiko and Westerlund, 2013) *FliC* flagellin is an vital part of chemotaxis of bacteria, during infection, bacteria used it to epithelial cells attached by binding with asialyated glycolipid asialoGM1 and can produce a strong nuclear factor kappa-light-chain enhancer of activated B cell NF-kB-mediated inflammatory response through signaling through TLR5 and a caspase-1-mediated response through the Nod-like receptor (NLR), Inter-leukin-1β-converting enzyme (ICE) ICE protease-activating factor (Ipaf) (Miao *et al.,* 2007) the Ipaf inflamma some responds to the presence of cytosolic flagellin. Caspases are evolutionarily conserved cysteine proteases that induce apoptosis. There are no any significant role of Caspases -1, -4 and -5 include the human inflammatory caspases in apoptosis (Creagh *et al.,* 2003) Instead of that, it is (caspases-1, -4 and -5) play essential roles in inflammatory processes and is very important for the mature forms production of some inflammatory cytokines in response to infectious agents and to the sterile injury (Li *et al.,* 2009). The role of the inflammatory caspases-5, is

increasingly being recognized in pyroptosis. Originally identified as ICErelII, in addition to being called ICH-2/ Transcription factor X (TX), caspase-5 were iden-tified as cysteine proteases with 52% homology to caspase-1, and over 75% homology to each other (Kamada *et al.,* 1997).

While structurally similar to caspase-1, caspase-4 and 5 have lower affinity to caspase-1 specific substrates (Fassy, 1998). Caspase-5 was identified as being part of the original NLRP1 inflamma some complex and caspase-4 were also identified as being critical for caspase-1 activation (Sollberger, 2012). However, the precise role for these proteases is still being defined and it is proposed that they are upstream initiator caspases which facilitate caspase-1 activation (Thornberry, 1997) The caspase-5 secretion are induced by lipopolysaccharide (LPS), it is common activator for 1β and 18 immature ILs; thus, in related to human these caspases are involved in immunological responses (Parrish *et al.,* 2013).

The purpose of the study was to link the infections with the bacterium *P. aeruginosa*, which possesses the *flic* gene for the virulence factor of the astronomy and the activation of caspase-5.

MATERIALS AND METHODS

Study Design and Population: Seventy three blood sample and isolates were collected, fifty- suspected as *P. aeruginosa* were collected from different clinical samples, using sterile swabs, they were from various sources, the source of these isolates which are (n=12) urine cultures, (n=10) sputum, (n=9) wounds, (n=19) burns and (n=3) external otitis from hospitalization patient from different Baghdad hospitals from (October 2016 till April 2017).

Sample Collection

Bacterial isolate: The samples of swab has been taken from different patients those suffering from infected wounds like, infected burn, ear and wound infections. In addition of sputum and urine samples collected from different infections.

***P. aeruginosa* phenotypic identification:** The phenotypes of bacterial isolates detected by culture all swaps in brain-heart infusion broth, according to Chess rough (1991) are plated on to MacConkey agar, nutrient agar, blood agar. Then in selective media (cetrimide agar and pseudomonas agar). Then the *P aeruginosa* phenotyping detected using Gram stain and pigment production after incubation at 37ºC and accordingly the biochemical test was done (MacFaddin, 2000) that include: Oxidase and catalase tests, then for biochemical test the (API 20E System) used that consists of 20 micro tubes, containing dehydrated substrates.

Antimicrobial susceptibility test: The *P. aeruginosa* isolates antimicrobial susceptibility was studied by using disk diffusion method (Ferraro *et al.*, 2000). Antibiotic impregnated discs (Bioana-lyse, Turkey) containing 24 different antibiotics were used showed in table 3-2.

Quantitative Detection of biofilm formation: The ability of Biofilm-forming was estimated by adhesion determination to microtiter plates according to Bose, *et al*., (2009) in briefly, isolate incubated in Luria-Bertani (LB) for 18 hours at 37°C. 300µL of LB spread on flat-bottom with 10µL of bacterial broth, then plates incubated for 18 hr at 37 °C then plates washed and air-dried at 60°C for 1 hr. it stained by crystal violet for 1 min (300µL, 0.25%) after washing the acetic acid (300µL, 33%) was used to de-stain, then OD for each well of plates were measured at 570nm. All tested were conducted three times and the average of results were considered (Bose *et al.,* 2009).

Sample collection of blood: Samples 5 ml of blood were collected from patient that are suffering from *P. aeruginosa* infections for DNA isolation (Molecular genetic studies)**.**

DNA Extraction

Genomic DNA extraction: By using a wizard Genomic DNA Purification Kit Genomic DNA of Bacteria was extracted (Promega, Madison, WI). as per the manufacturer instructions (Promega, USA): Cell Harvesting : transferred the bacterial culture to a 1.5 ml micro centrifuge tubes, centrifuged for 2 minute at 13-16000x g and then discarded the supernatant. DNA extract according to manufacture protocol. The supernatant was stored at -20◦C as a template DNA stock.

Genomic DNA of blood was extracted by using the Genomic DNA purification Kit for Human Blood as per the manufacturer instructions (Promega, USA): 300µl Sample Volume from fresh whole blood was taken from the patient. DNA extract according to manufacture protocol. The supernatant was stored at -20◦C as a template DNA stock. The DNA amount and purity were measured by spectrophotometer (Eppendorf, Germany). Finally, the pellet was dissolved in TBE buffer.

Polymerase Chain Reaction: A Fifty *P. aeruginosa* samples were selected for PCR analyzing. The mixture reaction of PCR was consist of (12.5µl of GoTaq®Green Master, template DNA 5µl, forward and reverse primers 1.25µl and 5 µl of dH2O) thermal Cycler (Gene Amp, PCR system 9700, Applied Biosystem). The primers used as *16S rDNA0*, *PA-SS*-F and *PA-SS*-R was used (Spilker *et al.*, 2004), which amplifies 956bp. The amplification program was run as follow: 1 /cycle of 95°C for 2 min, 30 cycles (92°C 1 min,55 - 59°C 1 min then

72°C I min) final extension 72°C for 10 min. for Detection virulence genes flagellin gene and its sub type *Flic* A, *Flic* CW45 and CW46 Primer pair *flic*-F- CW45 (5' GGCAGCTGGTTNGCCTG 3') 1.02 kb (type a) *flic*-R-CW46 (5' GGCCTGCAG ATCNCCAA 3') 1.25 kb (type b), the amplification program was run as 95°C for 2 min, 30cycles (95°C 40 sec, 55°C 1 min and 72°C for 2 min) final extension 72°C for 10 min. the primer of *Casp-5* for caspase-1 gene, F (5' GATATGGAGTCA-GTGCTGAG 3') and *casp-5*-R (5' CTGCAGGC-CTGGACAATGA 3') 230bp, the amplification program was 95°C for 3min, thirty cycles (94°C 30sec, 54°C 20sec and 72°C for 20 sec) and 1 final extension 72°C for 5 min. Then the product of amplify was running in 1.5% agarose gel electrophoresis which stained by used ethidium bromide.

DNA Sequencing: Sequencing of amplified product of *casp-5* gene was done by (National Instrumentation center for Environmental management (NICEM) through using forward and reverse primer for *casp-5* gene in sequencing reaction. Alignments implemented by BLAST version 1.1.0 and BioEdit program. The result was compared with reference sequence of the gene obtained from gene bank as control.

RESULTS AND DISCUSSION

P. aeruginosa is found widely distributed in the environment and is considered an opportunistic pathogen. The present study selected 50 clinical *P. aeruginosa* isolated from hospitalization patients from October 2016 to April 2017. Using the API 20E (bioMérieux) and conventional biochemical tests, *P. aeruginosa* strains isolated from the different clinical sources were identified: 38% (19/50) from Burn's, 24% (12/50) from urine, 20% (10/50) from sputum, 12% (6/50) from wound and 6% (3/50) from otitis. Seventy three isolates were cultured on macConkey agar, nutrient agar, pseudomonas agar and citrimide agar. Only 50 *Pseudomonas aeruginsa* grow after 24h in 37◦C based on biochemical test while other 23 isolate not *p. aeruginosa*. From 50 isolate (48) from fifty isolates gave a positive results on citrimide agar that containing nalidixic acid, it is a useful antibiotic for isolation and identification of *P. aeruginosa* from other species of *Pseudomonas*, because it resists against this antibiotic but others show sensitivity against it (Kodaka *et al.*, 2003) And API 20E test recorded positive results for all types of isolates with just 3(6%) isolates from wound this infection may be caused by some mucoid strains of *P. aeruginosa* (Fadhel, 2013) which are difficult to distinguish by API 20E test table 1 .

Table -1: The results of cultural tests on different media and API 20E test

Type of isolates	MacKonky agar	Pseudomonas agar	Blood agar	Citrimide agar	Nutrient agar	API 20E test
otitis	3/3	3/3	3/3	1/3	3/3	3/3(100%)
urine	12/12	12/12	12/12	12/12	12/12	12/12(100%)
wounds	6/6	6/6	6/6	6/6	6/6	3/6(50%)
burns	19/19	19/19	19/19	19/19	19/19	19/19(50%)
sputum	10/10	10/10	10/10	10/10	10/10	10/10(100%)

Antimicrobial susceptibility test: The results showed in Table 2 isolates a different ability to resist 24 antibiotics.

Table -2: Results of susceptibility tests of *P. aeruginosa*

symbol	Antibiotics	Number of bacterial isolates and their percentege		
		R	I	S
AK	**Amikacin**	21(42%)	4(8%)	25(50%)
AMC	**Amoxicillin- clavulanic acid**	50(100%)	-	-
AMP	**Ampicillin**	36(72%)	-	14(28%)
ATM	**Aztreonam**	34(68%)	7(14%)	9(18%)
CAZ	**Ceftazidime**	14(28%)	-	36(72%)
CIP	**Ciprofloxacin**	28(56%)	-	22(44%)
CLT	**colistin**	47(94%)	-	3(6%)
CPM	**Cefepime**	45(90%)	-	5(10%)
CTR	**Ceftriaxone**	37(74%)	2(4%)	11(22%)
CTX	**Cefotaxime**	44(88%)	-	6(12%)
ERY	**Erythromycin**	50(100%)	-	-
GEN	**Gentamycin**	43(86%)	2(4%)	5(10%)
IPM	**Imipenem**	15(30%)	5(10%)	30(60%)
LEV	**Levofloxacin**	12(24%)	-	38(76%)
MEM	**Meropenem**	1(2%)	-	49(98%)

NA	**Nalidixic acid**	48(96%)	-	2(4%)
NiT	**Nitrofurantoin**	9(96%)	1(2%)	1(2%)
PG	**Penecillin G**	48(96%)	-	2(4%)
PI	**Piperacillin**	31(62%)	3(6%)	16(32%)
TCC	**Ticarcillin-clavulanic acid**	37(74%)	-	13(26%)
TE	**Tetracycline**	49(98%)	-	1(2%)
TI	**Ticarcillin**	38(76%)	4(8%)	8(16%)
TOB	**Tobramycin**	39(78%)	-	11(22%)
TS	**Trimethoprim-sulfamethoxazole**	50(100%)	-	-

Amoxicillin- clavulanic acid, Trimethoprim- sulfamethoxazole and Erythromycin were the effectiveness antibiotic against *P. aeruginosa* 100%.

Estimation of Biofilm formation quantitation by the microliter plate method (MTP): The present research showed different ability to form biofilm by using MTP method as shown in Table 3.

Table - 3: Biofilm results of *P. aeruginosa* in MTP method

Biofilm formation	**Percentage% and number**
High producer	76%(38/50)
Moderate	16%(8/50)
Non-producer	8%(4/50)

The results of the present study found that most of the isolates have ability to produce biofilm were resistant to antibiotics. These results were consistent with (Kaur and Wankhede, 2013). They were found that 65% of the isolates resistant to antibiotics have the ability to form the biofilm, unlike the isolates that cannot form This biofilm indicates the importance of the biofilm and its role in the emergence of highly antibiotics resistance by many of bacterial species that produce it (Bacalso *et al.*, 2011) that help it to adhere to host cells (Vallet *et al.*, 2004) and gave it the protection of bacteria from external conditions is not appropriate, which helps to stay on hard surfaces, especially in the hospital environment and this leads to the occurrence of injuries acquired from hospitals (Nosocomial infections) (Ramos *et al.*, 2013).

Genotypic identification

Genomic DNA isolation from bacterial isolates: The result was found that, by using this protocol, for extraction of *P. aeruginosa* DNA, the full amount of obtaining DNA (Figure 1) was very efficient method, so we obtained a good yields of genomic DNA.

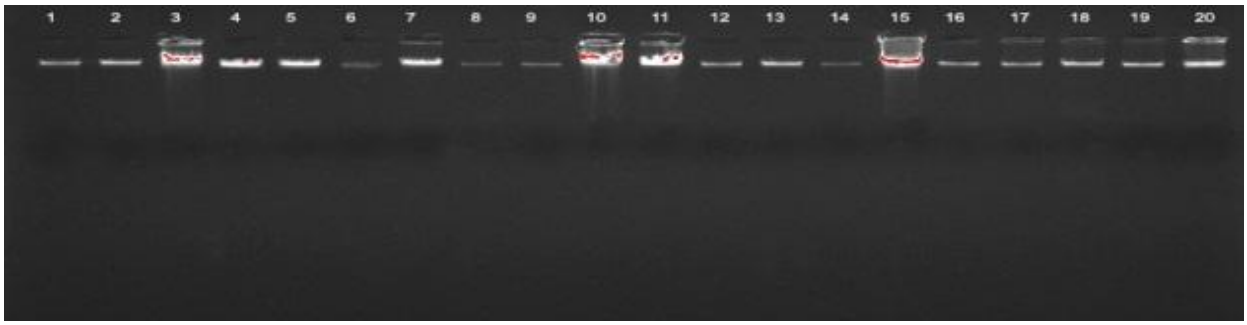

Fig - 1: Agarose gel electrophoresis of bacterial genomic DNA using 0.7% agarose, TBE (1x) and visualized by ethidium bromide stain.

PCR Analysis for bacterial isolates: In this study two pairs of primer was used; (pair Pa SS-F, Pa SS-R) that were specifically for *P. aeruginosa*. The target of that primers in the 16S rRNA gene are variable regions. Assays of PCR that using this this primer pair produced a predicted size of DNA products (Fig. 2). A useful identification method of bacteria has been offered by *16S rRNA* gene sequence, which was for long period used as method of taxonomic for determining the bacterial species phylogenies (Drancourt *et al.*, 2000) the results showed all 50 isolates gave positive results.

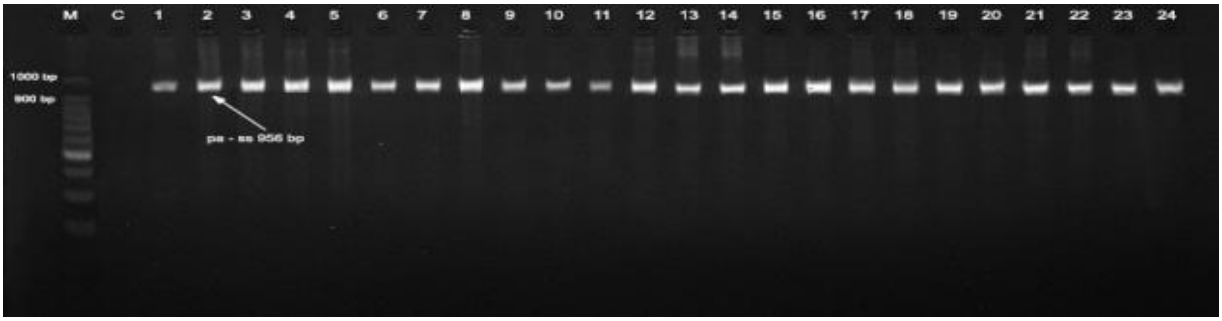

Fig. -2: 956 bp PCR products of *16S rDNA* which was specific for *P. aeruginosa* were identified in all samples in 1.5% (1 h /70 vol) agarose gel electrophoresis. M 1500kb DNA ladder, c: Negative control and 1- 24 were various samples of *P. aeruginosa* isolates.

The *flic* gene asserts that the bacteria are contained on the virulence gene of activating and caspase-1 which in turn leads to the Interleukin-1 beta production. *Flic* gene is consist of two types: type A, which has a molecular weight of 1020 base pairs and type B 1250 base pairs (Faezi *et al.*, 2006).

The results of our research showed all isolates were positive, 28(56%) isolate had type a and 17(34%) isolate had type b of the gene, but 5(10%) there were isolates that did not have a flic gene, these were isolates which were cystic fibrosis infection 4 sample (Fig. 3) and 1uti infection of isolate this is agreement with Allison (1985).

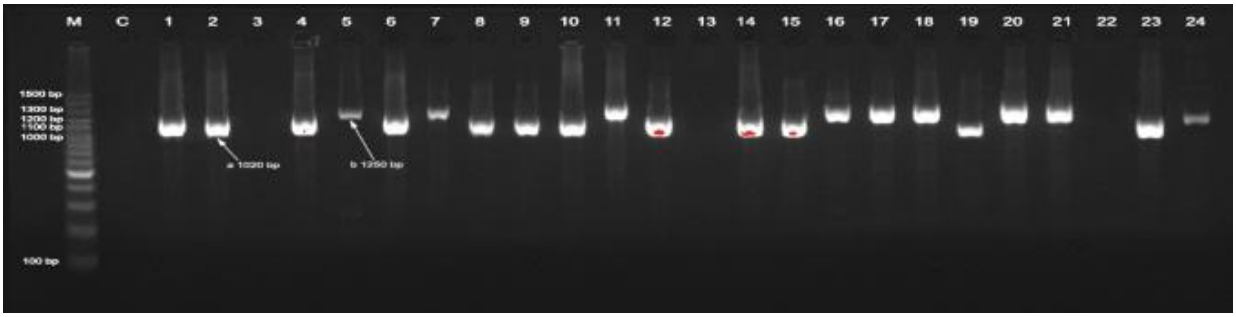

Figure 3: Agarose gel electrophoresis of PCR product amplified from *flic* gene, the DNA fragments of type a 1020 bp and typeb 1250bp, were amplified from *flic* gene using ladder; lane M (100-1500 bp),lane C is control negative. The figure shows all samples gave positive result (lanes from 1 to 24) except five isolates gave negative results.

Except the factors of extracellular, the flagella (initial attachment mediator) significantly, playing a role in infection initiation. In *P. aeruginosa*, there were 2 types of protein in flagellin, which had been identified as type 'a' and type 'b' protein (Fig. 4), that can be recognized from each other according to the molecular size basis and type-specific polyclonal and monoclonal antibodies reactions (Allison *et al.*, 1985) both flagellin types of *P. aeruginosa* were not exhibit phase variation; a single flagellin type was produced by a single strain and there were no observed of switching among these types.

The primers of flagellin gene PCR PAO1, were specific for N-terminal (CW46) and C-terminal (CW45) the analysis of *fliC* locus sequencing revealed heterologous groups of a-type (1164bp; 1185bp) and highly conserved 'b'-type (1467bp) flagellin genes. The Occurrence percentage of *fliC* was 90% (37.77% '*b*'-type flagellin, 62.23% '*a*'-type) (Lena *et al.*, 2016).

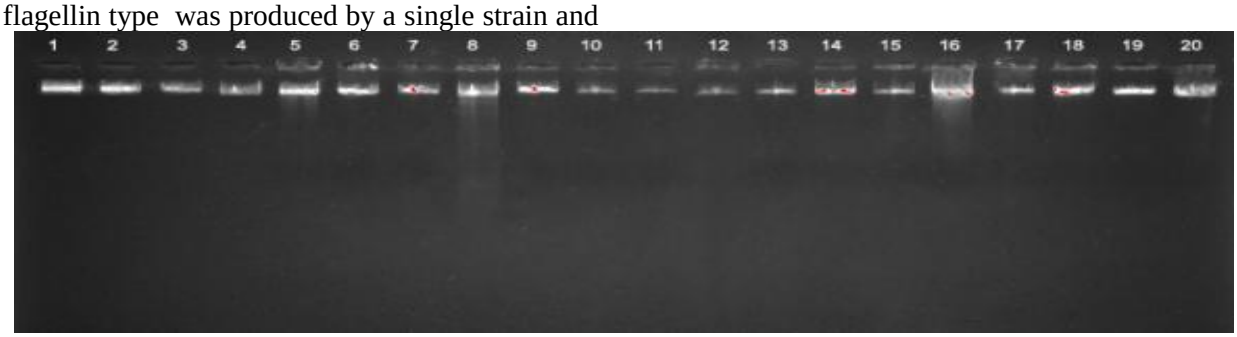

Fig. -4: Agarose gel electrophoresis of human genomic DNA using 0.7% agarose, TBE (1x) and visualized by ethidium bromide stain.

Genomic DNA isolation from human blood: The study of caspase-5 in terms of inflammatory and stimulate the immune system, according to our research we think we are the first studied in this field and studied the relationship between caspase-5 and *p. aeruginosa* infection. The result for the *casp-5* gene was positive for all isolates as shown in Fig. 5.

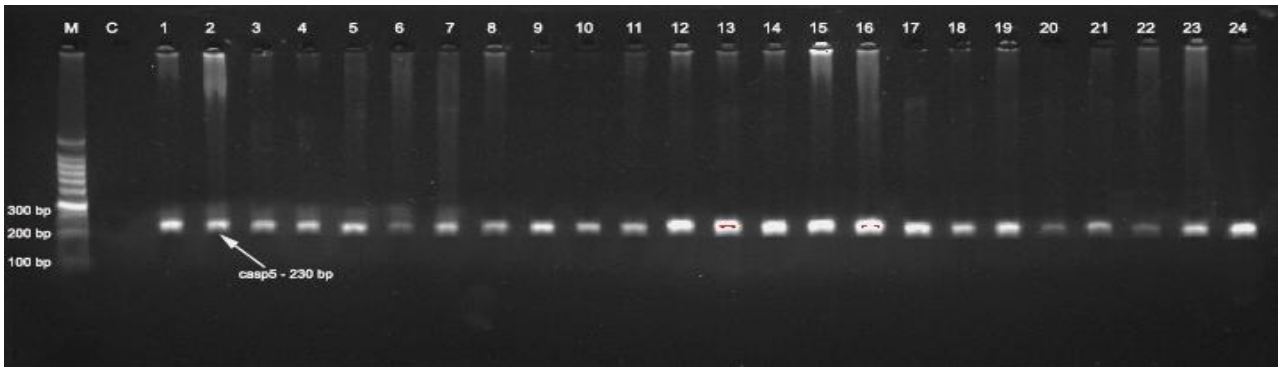

Figure 5: Agarose gel electrophoresis of PCR product amplified from *casp-5* gene, the DNA fragments of 230bp were amplified from *casp-5* gene using ladder; lane M (100bp), lane C is control negative. The figure shows all samples gave positive result (lanes from 1 to 24)

***Casp-5 gene* Sequencing:** Twenty amplified products of *casp-5* gene direct sequencing for detecting SNPs within these sequences then compared with reference sequence of *casp-5* in national center biotechnology information (NCBI) Gene Bank. Primer set covers exon count 10 on *Casp-5* gene. The number of samples in which we found a genetic variance were 20 from 20 samples (Fig. 6). Had a heterozygous nucleotide. This is the first study to our knowledge to give gene variation of inflammatory caspase-5 in Iraqi bacterial infection patients.

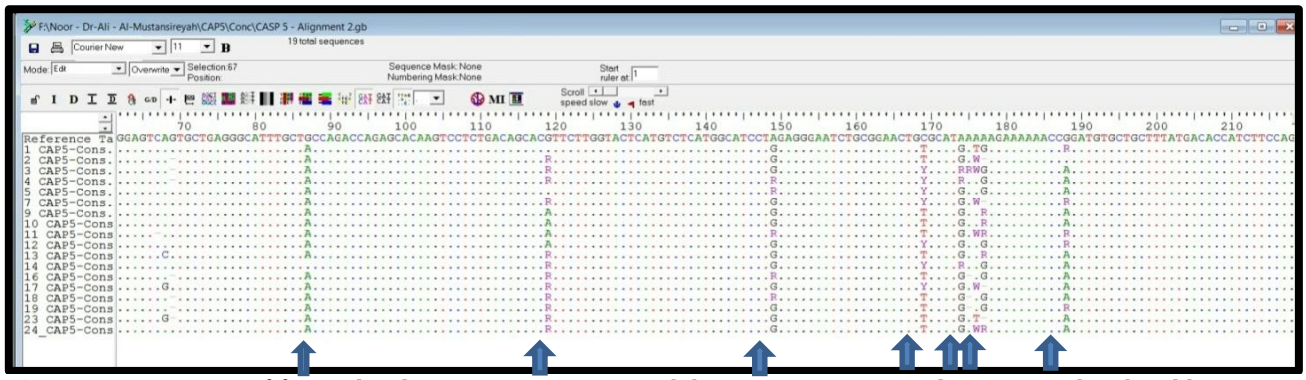

Figure 6: Sequencing of forward and reverse *casp-5* gene with heterozygous R, W and Y compared with wild type *casp-5*obtained from Gene Bank.

The most common type of human genetic variation are SNPs, which accounting for two to three cm of the human genome (Jin *et al.*, 2015) In addition, protein expression or structure directly affect by SNPs which exhibit a high genetic stability, therefore underlie numerous genetic disorders (Chen *et al.*, 2013)

The immunity response variation is genetically controlled and an inflammatory pathway is associated with it, specifically the influence of the diseases outcome (Noreen and Arshad, 2015) the infection genetic susceptibility or even sepsis can be affected by (SNPs) of casp-5 (Schnetzke *et al.*, 2015).

So the responses of influence of the innate immune towards challenges of pathogenic and outcome of disease are greatly affected by variations of genetic such as (SNPs); therefore, there is a susceptibility range appears among people to infections, some of them are being protected against infection while others are being predisposed to infected (Skevaki *et al.*, 2015).

The results of our research showed a high genetic variability in the *casp-5* gene, where 20 from 20 samples had heterozygous gene-altering sequences in the nucleotide sequences (Qing *et al.*, 2009).

Conclusions

The results showed a different ability to resist antibiotics from isolates. Amoxicillin- clavulanic acid, Trimethoprim-sulfamethoxazole and Erythromycin were the effectiveness antibiotic against *P. aeruginosa* 100%. The present research showed different ability to form biofilms by using MTP Method, 46(92%) was former (strong and moderate producer).

The results of Genotypic detection for virulence gene (*Flic* gene) showed that the 45(90%) were positive then the isolates. so, we concluded that the isolates that have the virulence gene are very effective of caspase-5 activation. Primer design for human *casp-5* gene enzyme % 100 samples were positive. Sequencing for 20 PCR product of *casp-5* gene to detect variations of Iraqi population 20 of it have a heterozygous nucleotides.

When the study was completed and the results were collected, the results were interrelated as the bacterial isolates that were strongly resistant to antibiotics had high virulence factors, such as biofilm formation. The isolates possessed the *flic* gene responsible for activating the caspase-5. All these results confirmed by the result of the sequencing, where the samples conducted by the test of the sequence were highly heterogeneous genetic sites of certain gene and this result was compared with the reference sequence of caspase-5 approved by NCBI.

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