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Molecular diagnosis of bacteria *Pseudomonas aeruginosa* isolated from urinary tract infection in women and detection of virulence factors

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Abstract
The results of the research showed the isolation and diagnosis of *Pseudomonas aeruginosa* bacteria from a woman with urinary tract infection, and she reviewer for Al-Khansa Teaching Hospital. The bacterial isolation was diagnosed based on microscopic diagnosis, phenotypic characteristics and biochemical tests, as well as molecular diagnosis was made through PCR technology and based on the 16S rRNA gene as well as Matching the sequences of the genes with the sequences documented in the National Center for Biotechnology Information (NCBI). A new gene was obtained in the bacterium *Pseudomonas aeruginosa* in addition to being registered as Pseudomonas aeruginosa ZH-Almola in the (NCBI).

Then, a sensitivity test was conducted for the most commonly used antibiotics for urinary tract infections, 10 types of antibiotics were used, and Ciprofloxacin was the most effective antibiotic.

Important virulence factors were also investigated, as five virulence factors were studied (hemolysin enzyme, urease enzyme, protease enzyme, the ability to form biofilms, and the ability to produce beta-lactamase enzymes), The results showed their ability to produce most of the important virulence factors, as the bacteria was a producer of hemolysin enzyme and urease enzyme and capable of forming biofilms and beta-lactamase enzymes, while it was not producing protease.

Keywords: *Pseudomonas aeruginosa*, Molecular, women, factors

Introduction

The urinary system is one of the important organs in the body, and any defect that occurs in its function can affect the rest of the organs due to its role in regulating the volume of cellular fluid and its components [1]. Urinary tract infection is one of the inflammatory diseases that result from the high multiplication of many pathogens in the urinary system, which leads to changes in the optimal function of the urinary tract and kidneys [2]. The presence of large amounts of pathogenic bacteria in urine is indicative of a UTI, despite the presence of a number of different defense mechanisms of the host body against this bacterial infection in the urinary tract [3]. And that urinary tract infection is a clinical problem that constitutes (1-6%) of medical referrals in hospitals for urinary tract infections and infections of the bladder and kidneys [4].

UTI occurs at all ages and of both sexes, but in females it is higher than males, for several physiological reasons, including: the shortness of the urethra and its proximity to the anus, hormonal activity, and because of the bacteria of the digestive system that pollute the area surrounding the rectum, infect the urethra and spread to the bladder [5], The infection is caused by Gram-negative and Gram-positive bacteria, as well as fungi, viruses and parasites, and the most common types of bacteria that infect the urinary tract and cause infection are (*Escherichia coli*, *Proteus spp*, *Klebsiella spp*, *Pseudomonas spp*, *Staphylococcus spp*.) The severity of infection depends on the virulence of the bacteria and the susceptibility of the host [6].

Virulence factors are molecules secreted by bacteria and play an important role in the infection and pathogenesis of bacteria, such as colonization of host cells, cell adhesion and invasion of the host immune system, as well as inhibiting the immune response, as there are a number of virulence factors possessed by bacteria that play an important role In bacterial pathogenesis [7], One of the most important factors of virulence is the ability of pathogenic bacteria to produce hemolysin, a cytolytic enzyme that degrades red blood cells by creating holes in their cell membranes [8], The enzyme urease is one of the important factors, which is produced by pathogenic bacteria that cause urinary tract infection and cause the formation of kidney stones and block urinary catheters. The protease enzyme is also an important factor, as it causes tissue destruction through the decomposition of protein materials in tissues, as well as causes skin necrosis in skin injuries [9].

The ability of pathogenic bacteria to produce biofilm cell membranes is one of the most important factors of virulence, which helps them to resist antibiotics [10], as well as their ability to produce β-lactamase enzymes that help them to resist beta-lactams, as it works to break the beta-lactam ring necessary for the action of the antibiotic [11].

Materials and methods

the sample

A urine sample was taken from a woman who was referred to Al-Khansa Teaching Hospital, who has urinary tract infection and is pregnant in the seventh month, then it was kept in a special sterile collection tube, Then it was transferred to the Microbiology Lab at the College of Education for Pure Sciences / Department of Life Sciences / University of Mosul, as soon as possible for the purpose of Implantation, diagnosis and study.

Culture

The sample was cultured on plates containing nutrient agar medium, blood agar medium, MacConkey agar medium and Cetrimide agar medium prepared previously, by means of a loop and by the planning method. The dishes grown in the incubator were incubated at a temperature of 37 °C for a period of 24 hours, and kept in the refrigerator at a temperature of (4) °C until biochemical diagnostic tests were carried out for them, and the PCR technique was used to diagnose them molecularly.

Antibiotics

(10) types of antibiotics were used and prepared by Bioanlysis Company, as in the following table:

Table 1

NO.	Antibiotic	Symbol	µ
10	Gentamicin	CN	10
10	Chloramphenicol	C	10
15	Azithromycin	AZM	15
5	Cefixime	CFM	5
10	Amoxicillin / Clavulanic acid	AMC	10
10	Ceftriaxone	CRO	10
25	Ampicillin	AM	25
10	Clindamycin	DA	10
10	Penicillin G	P	10
10	Ciprofloxacin	CIP	***10***

Diagnosis

The diagnosis was based on phenotypic characteristics (on culture media), microscopic examinations (by dyeing it with Gram stain), biochemical tests, and also the use of molecular diagnostics using the (PCR) machine to diagnose the isolate of Pseudomonas aeruginosa bacteria, and the results that appeared were recorded.

Phenotypic characteristics

The bacterial isolate was initially diagnosed based on the appearance of the colonies in terms of their shapes, color, texture, transparency, sharp or high surfaces of the colonies, as well as the presence of distinctive odors and the type of decomposition on the blood agar medium, as well as the fermentation or non-fermentation of lactose sugar on MacConkey agar medium, in addition to its ability to grow on Cetrimide medium for *P. aeruginosa* [12, 13].

Microscopic diagnosis

P. aeruginosa was examined microscopically after staining it with gram stain and examined under a microscope to observe its shape and arrangement of its cells as well as its interaction with the dye in terms of color [12].

Biochemical test

A group of biochemical tests were conducted for the purpose of diagnosing *P.aeruginosa* (catalysis test, oxidase test, citrate utilization test, indole test, motility, gelatinase enzyme test, vogas-Proskauer test, methyl red test). [14, 15, 16, 17].

Molecular Diagnosis

Molecular techniques were used to diagnose P. aeruginosa through PCR technology and using the 16S rRNA gene, which is a diagnostic trait, and then the nucleotide sequence was compared with the sequence recorded in the database to obtain the name of the genus, species and strain [18].

Determining the nucleotide sequences of the amplified pieces using the sequencing technique

The nitrogenous base sequences of *P.aeruginosa* were sequenced by sending the PCR products for the 16s rRNA region of the sample with the primers of the resulting package. The gene sequence was read using the Genetic Analyzer (3130) And the equipment from Hitachi Corporation of Japan, and then the sequences of the genes were matched with the sequences of the genes documented in the National Center for Biotechnology Information NCBI, with the analysis of the results obtained using the (BLAST) program.

Antibiotic sensitivity test

The sensitivity of *P.aeruginosa* to the 10 antibiotics was tested using the Kirby_baure method, as stated in [19].

Detection of virulence factors

1: Detection of hemolysin enzyme production: *P. aeruginosa* bacteria were cultured on a dish containing blood agar medium prepared in advance, Then the cultured dish was incubated in the incubator at a temperature of (37)ºC for 24 hours, When a transparent halo is formed around the implantation area, this means that it produces the enzyme hemolysin and is capable of hemolysis [15].

2: Detection of urease production: *P.aeruginosa* was inoculated in the tube containing the urea agar medium, which was previously prepared by stabbing and plotting on the inclined surface, Then, the tube is incubated in the incubator for 24 hours at a temperature of 37oC, and when the color of the medium turns pink, this means that these bacteria are able to produce the urease enzyme [15].

3: Detection of the production of protease enzyme: This was done by inoculating the previously prepared sorting milk agar (Skimmed milk agar) with *P. aeruginosa* in a straight line in the culture medium, Then the dishes were incubated in the incubator at (37)º C for a period of (24) hours, and when a transparent area was formed around the culture line, it was evidence of the ability of these bacteria to produce the protease enzyme [17].

4: Detection of biofilm production using the Congo red agar method: *P. aeruginosa*, which is to be tested for its biofilm-forming ability, was inoculated on Congo red medium and incubated aerobically for 24 hours at 37 ºC, After the incubation period, it was detected if it formed biofilms by the appearance of the developing colonies on this medium in a dry or shiny black color, but if they were not formed, they appeared in a red or wine color [20].

5: Detection of the production of beta-lactamase enzymes using the acidic method: the reagent used in this method was prepared by taking (2) ml of phenol red solution at a concentration of (0.5%) w/v of distilled water, then (1.2 g) of penicillin G was added to it and the was set pH at (8.5), Then the test was carried out by taking (0.1) ml of the reagent and placing it in a clean and sterile ependorff, after which it was inoculated with *P.aeruginosa* bacteria that wanted to reveal its ability to produce beta-lactamase enzymes, that grown on the solid medium and it was possible to use the liquid medium as well, and a thick bacterial suspension was produced, Then the ependorff was left in the room at a normal temperature and it was observed that the color of the medium changed from red to yellow, as the appearance of yellow color is a positive result of this test [21].

Results and discussion

Phenotypic characteristics

Bacteria *Pseudomonas aeruginosa* appeared in a pale color that did not ferment lactose, with a luster when grown on MacConkey agar medium, but when cultured on a blood agar medium, it was beta-haemolysis, As a transparent halo appeared around the colonies, this indicates that they secrete the enzyme hemolysin, and to confirm the diagnosis, it was transplanted on the medium of the cetrimide, so the colonies appeared in a mucous, smooth shape and cream color, As well as from the diagnostic qualities, it has a clear blue-green tint when it grows on the Nutrient agar medium.

Microscopic diagnosis

P. aeruginosa appeared as medium-sized, Gram-negative bacilli.

Biochemical test

Pseudomonas aeruginosa gave a positive result in the catalase test, and the oxidase test result was positive, as it has the ability to produce the enzyme Cytochrome C oxidase, And it appeared that she possesses the gelatinase enzyme, and the result was positive for the gelatin test, positive for the movement test, and it gave a negative result for the indole test, The result of the Methyl red test was negative because it does not ferment glucose sugar, and it was negative for the Voges-Proskauer test as well. As for the citrate test, it was positive because of its ability to consume citrate as the only source of carbon, and the color of the medium changed from green to blue as a result of the change in pH for the middle.

Molecular Diagnosis

Molecular diagnosis was carried out using PCR technique, and this was done based on the diagnostic gene 16S rRNA, which is characterized by being a highly conserved region and little heterogeneity in the bacterial type, and the results showed that the sequence of the diagnostic gene of P. aeruginosa is 100% present in it

Determining the nucleotide sequences of the amplified pieces using the sequencing technique

The sequence of the nitrogenous bases of *P. aeruginosa* was determined. The PCR products of the 16S rRNA region were sent to this sample with the primers of the resulting package. The sequence was read for the genes based on the Genetic Analysis 3130 device supplied by the Japanese company Hitachi.

The sequences of the genes were matched with the sequences documented in the National Center for Biotechnology Information (NCBI) and the results were analyzed using BLAST program, and a new gene was obtained in P. aeruginosa. This gene has been registered in the NCBI Global Genetic Data Bank.

_ Sequence analysis of the 16S rRNA gene in isolate Bio2ab09 of Bacteria

GGACAGGCGGCATGCTACACATGCAGTCGAGCGG
ATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGAC
GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGG
GGGATAACGTCCGGAAACGGGCGCTAATACCGCAT
ACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACC
TCACGCTATCAGATGAGCCTAGGTCGGATTAGCTA
GTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCC
GTAACTGGTCTGAGAGGATGATCAGTCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGCGAAAGCCTGAT
CCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGA
TTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTA
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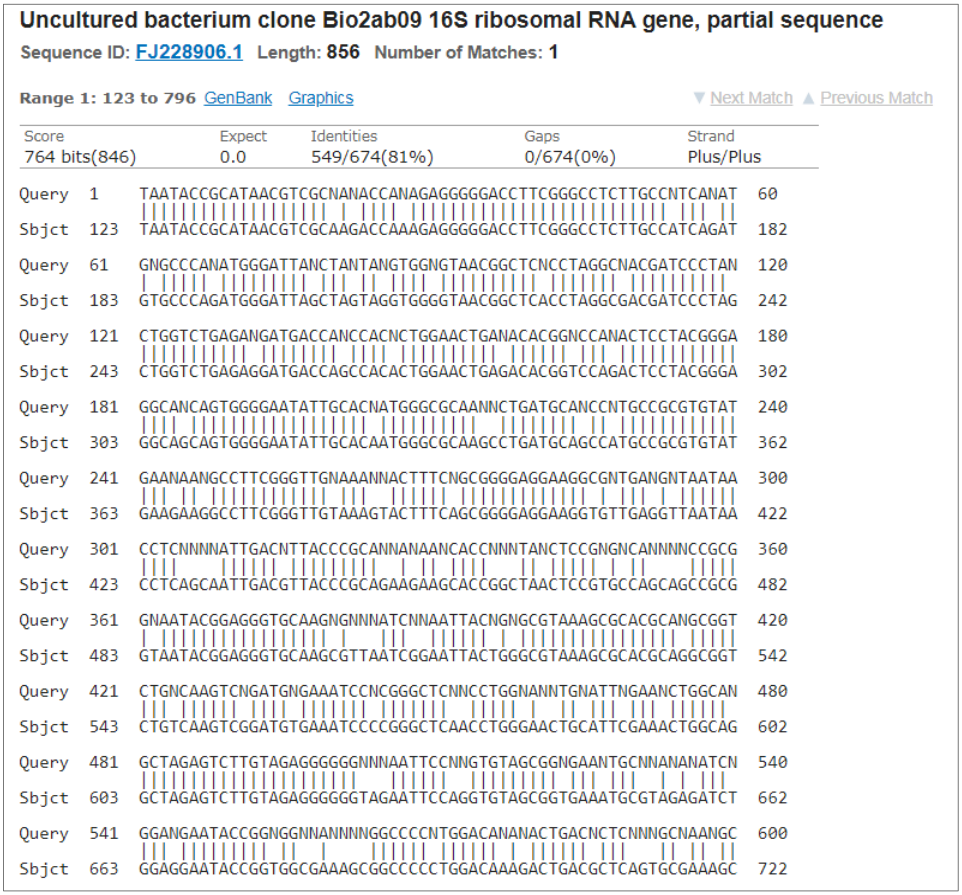

Uncultured bacterium clone Bio2ab09 16S ribosomal RNA gene, partial sequence

Sequence ID: FJ228906.1 Length: 856 Number of Matches: 1

Range 1: 123 to 796 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
764 bits(846)	0.0	549/674(81%)	0/674(0%)	Plus/Plus

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Sbjct  123  TAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGAT  182

Query  61   GNGCCCANATGGGATTANCTANTANGTGGNGTAACGGCTCNCCTAGGCNACGATCCCTAN  120
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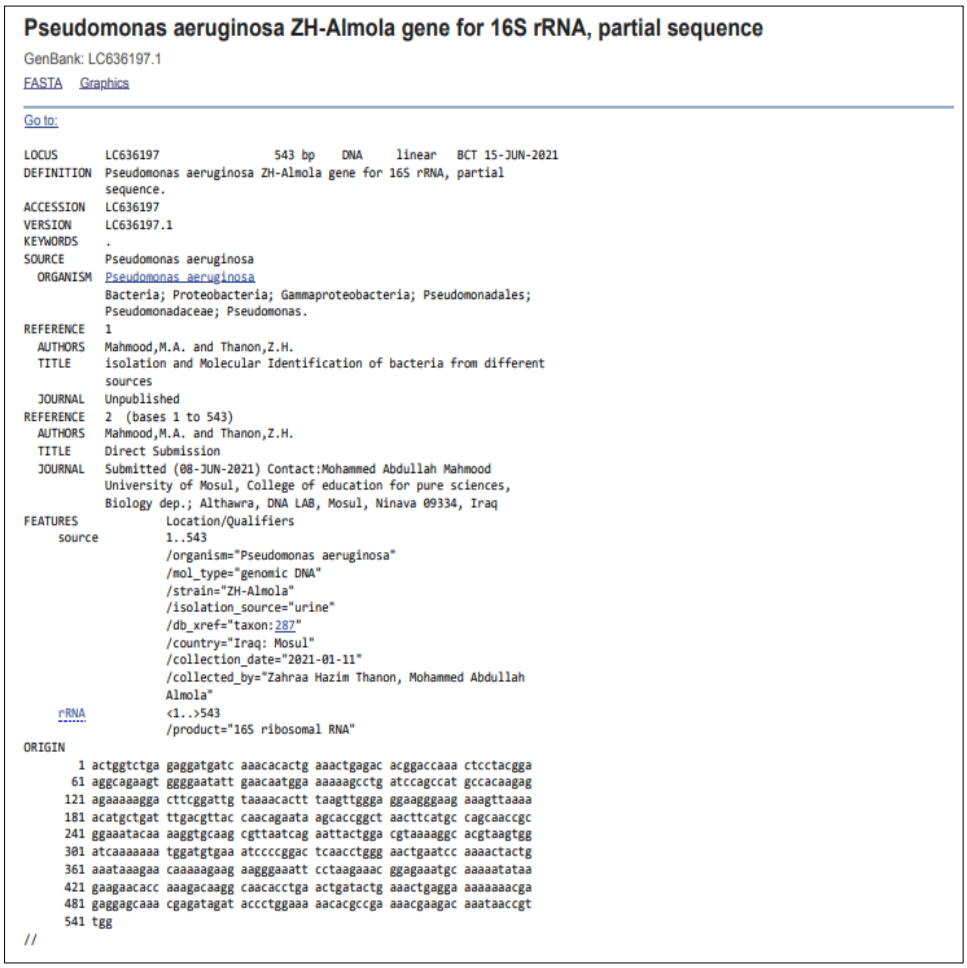
Fig 1



Pseudomonas aeruginosa ZH-Almola gene for 16S rRNA, partial sequence

GenBank: LC636197.1

FASTA Graphics

Go to:

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            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Mahmood,M.A. and Thanon,Z.H.
  TITLE     isolation and Molecular Identification of bacteria from different
            sources
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 543)
  AUTHORS   Mahmood,M.A. and Thanon,Z.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-JUN-2021) Contact:Mohammed Abdullah Mahmood
            University of Mosul, College of education for pure sciences,
            Biology dep.; Althawra, DNA LAB, Mosul, Ninava 09334, Iraq
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                     Almola"
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      121 agaaaaagga cttcggattg taaaacactt taagttggga ggaagggaag aaagttaaaa
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      301 atcaaaaaaa tggatgtgaa atccccggac tcaacctggg aactgaatcc aaaactactg
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      421 gaagaacacc aaagacaagg caacacctga actgatactg aaactgagga aaaaaacga
      481 gaggagcaaa cgagatagat accctggaaa aacacgccga aaacgaagac aaataaccgt
      541 tgg
//
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Fig 2

Antibiotic sensitivity test

P. aeruginosa showed resistance to four antibiotics (Cefixime, Ceftriaxone, Penicillin G, and Ampicillin), while it was sensitive to the rest of the antibiotics used in our study (Gentamycin, Chloramphenicol, Azithromycin, Amoxicillin/Clavulanic acid, and Clindamycin and Clindamycin).

Detection of virulence factors

1: Hemolysin enzyme: The results of the study showed that *Pseudomonas aeruginosa* bacteria have the ability to produce the enzyme β-hemolysin when planted on the medium of the blood agar. A transparent halo was observed around its colonies, and this indicates the secretion of the hemolysin enzyme that analyzed the blood, And this result was consistent with the findings of the researchers (22) who showed that it is capable of producing hemolysin enzyme and hemolysis. Hemolysin enzyme is an important virulence factor, It has the ability to degrade red blood cells, and the enzyme hemolysin, which is a poison, causes tissue damage, which facilitates the spread of bacteria and the release of nutrients to the host [23].

2: Urease enzyme: *Pseudomonas aeruginosa* gave a positive result for this test, which indicates its ability to produce urease enzyme, and this is consistent with what was stated (24) who showed that it has the ability to produce urease enzyme. The urease enzyme is one of the most important factors of virulence, as it plays an important role in the formation of stones by changing the pH of urine, which increases the deposition of ions in urine and thus increases the rate of stone formation [25].

3: Protease enzyme: The bacteria *Pseudomonas aeruginosa* gave a negative result in this study, as it was found that it was unable to produce the protease enzyme, As no transparent halo was observed around the implant line on the middle of the sorting milk agar, and casein was not hydrolyzed. This result was consistent with what the researchers found (26), The Protease enzyme is one of the important virulence factors responsible for destroying tissues and skin necrosis in skin injuries and bleeding internal organs in systemic injuries, as well as working to destroy tissues by analyzing protein substances, especially in muscle tissue [9].

4: Biofilms: *Pseudomonas aeruginosa* was strongly biofilm-producing, as the developing colonies appeared on the center of Congo red agar in black color, which indicates the positive test and the bacteria's ability to form biofilm. This result was in agreement with the findings of the researcher [27]. Biofilm is the binding and adhesion of microbial cells to living or non-living surfaces by means of materials that are complex organic polymers secreted by the microbial cells themselves to the outside of the cells to form the basic material on which they are based in order to create an effective, functional and independent ecosystem. Biofilm-producing bacteria are 1000 times more resistant to antibiotics than non-biofilm-producing bacteria [10].

5: Beta-lactamase enzymes: The result that we reached was that *P. aeruginosa* produces beta-lactamase enzymes. The color change after applying the reagent was observed from red to yellow, and this indicates that it possesses at least one of the beta-lactamase enzymes regardless of their types, because the acid method is a method revealed in general, This result is in agreement with the findings of the researchers [28]. It is one of the most important mechanisms that pathogenic bacteria use in resisting beta-lactam antibiotics, and this is done by breaking the beta-lactam ring necessary for the action of the antibiotic, which makes the antibiotic ineffective against bacteria [29].

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