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Molecular identification of some virulence and antibiotic resistance genes in *Pseudomonas aeruginosa* isolated from UTI infection

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THE study aim to isolates *Pseudomonas aeruginosa* from urinary tract infections using PCR, virulence gene, and antibiotic resistance detection using conventional and molecular methods. all sample of UIT for detection of *P. aeruginosa* were diagnosed by conventional ,biochemicals and VITEK-2 Compact technique; each isolate was cultivated on two plates of brain heart infusion agar, one used as a stock culture for antibiotic sensitivity test, while the other was used for genetic materials extraction and amplification of them to detect the presence or not of the virulence genes and the genes responsible for antibiotics resistance. The drug susceptibility test of 24 isolates of *Pseudomonas aeruginosa* was studied by the classical disk diffusion method against [12] antibiotics on Mueller-Hinton agar. The percentage ranged between [12.5%] imipenem and [100%] ampicillin. The molecular confirmation revealed that all 24 isolates were *P. aeruginosa* and came by the results of the VITEK-2 Compact technique. The results of molecular detection of virulence genes showed that 83.33% [20/24] of isolates were positive for the presence of the *algD* gene, in contrast, 70.83% [17/24] of them proprietor *toxA* gene. According to the outcomes of molecular identification of antibiotics resistance genes, the overwhelming majority of isolates carried the CTX-M gene [91.66%], and 75% of them were bearer SHV gene. In comparison, the TEM gene appeared in 45.83% of isolated *P. aeruginosa*. In conclusion, *P. aeruginosa* has the genetic weapons for considering and persisting infections, the bacteria own antibiotic-resistance genes that construct resistant bacteria which makes the cure and control of infection very hard.

Keywords: Molecular idntification , antibiotic, resistance genes, *Pseudomonas aeruginosa*.

Introduction

Pseudomonas aeruginosa infections often occur as hospital-acquired infections [1]. Infection is common in several conditions including respiratory infections, infections of the urinary tract, eczema, skin infections, arthritis, gastrointestinal infections, and several diseases [2].

More than 51,000 clinical infections with *Pseudomonas aeruginosa* are reported each year with 400 deaths annually in the United States according to the Centers for Disease Control [3].

Pseudomonas aeruginosa has been recognized as a main reason for nosocomial infections due to its widespread antibiotic resistance [4]. These anaerobic, facultative, Gram-negative bacteria inhabit a wide range of environments including catheters and medical equipment [5]. It is one of the common diseases in intensive care units [ICUs]. In addition, it is the main factor in infections that threaten the health and lives of burned people [6]. They have weak immune systems and are vulnerable to infections that can *Pseudomonas aeruginosa* is a major contributor to clinical infections worldwide, particularly in patients admitted to critical care units recovering from wounds, burns, trauma, and pre-existing lung conditions such as cystic fibrosis [7].

Numerous virulence factors possessed by *Pseudomonas aeruginosa* play a direct or indirect role in pathogenesis. These include ligand class, hemolysin, biofilm, elastase, exotoxin A, and proteolytic enzymes [8]. The synthesis of a large portion of cell-associated and secreted substances such as proteases and various toxins is essential for the *Pseudomonas aeruginosa* infection strategy [4]. These bacteria can become resistant to antibiotics because of genetic mutations or the acquisition of resistance genes from other bacterial species, which makes it difficult to treat the infections caused by

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these germs [9]. In addition, these bacteria produce β- lactamase enzymes and the production of Extended-Spectrum β- lactamase [ESBLs] produced by bacteria plays an important role in their resistance to many antibiotics [10]. the study aimed to confirm the diagnosis of *Pseudomonas aeruginosa* isolates by PCR technology using the *16S rRNA* gene, and detection of some virulence genes [Tox A, algD] and several genes that are responsible for antibiotic resistance.

Material and Methods

Twenty-four isolates of *Pseudomonas aeruginosa* that were diagnosed by conventional, biochemicals and VITEK-2 Compact technique were cultivated on brain heart infusion agar; each isolate was culture on two plates, one used as a stock culture for antibiotic sensitivity test, while the other was used for genetic materials extraction and amplifying.

Antibiotic sensitivity test [Kirby-Bauer Disc Method]:

The fresh broth culture was spread on Mueller-Hinton agar and allowed to dry for 2-5 minutes, then 12 antibiotic discs [Bioanalyse Co.- Turkey] were put on the surface of agar plates and incubated at 37°C\ 24h. diameter zone of inhibition measured by a ruler. The isolates were classified into 3 classes [Resistance, intermediate sensitivity, and sensitive] according to the Interpretation of zones of inhibition supplied by the manufacturing company of discs.

Genetic materials extraction and amplifying:

The extraction and amplifying were done according to[Presto™ Mini gDNA Bacteria Kit/ Geneaid Biotech. Ltd. Turkey The purity and concentration of the extracted DNA were measured by Implen NanoPhotometer®

Amplification processes:

Six couple of primes were used in the existing study [A couple for each gene] [Table 1]. The reaction mixture was composed of 12.5µl master mix [Promega GO Taq master mix], 2.5µl forward primer 10pm, 2.5µl reverse primer 10pm, 5µl DNA templet and 2.5µl PCR water [Total volume 20]. The amplification steps varied for each gene under study as in Tables [2-6].

Step of Electrophoresis:

The electrophoresis gel was done by solvent 2g of agarose in 100 ml of TBE solution [Promega] with adding 5µl safe dye [boiadd]. each hole of gel was loaded with The electrophoresis mixture consisted of 3µl amplicon DNA + 9 µl of loading dye [Promega], and one hole was loaded with 100bp marker [Promega] The Analytica gene Transilluminator was used for the bands' portrayal.

TABLE 1. Sequences of primers in the existing study

No.	Name of Primer	Sequence	Reference
1	*16S rRNA*	Primer-F: AGAGTTTGATCCTGGCTCAG Primer-R: CTACGGCTACCTTGTTACGA	Noor El-Deen *et al.* [11]
2	*AlgD*	Primer-F: ATGCGAATCAGCATCTTTGGT Primer-R: CTACCAGCAGATGCCCTCGGC	Benie *et al.*
3	*ToxA*	Primer-F: GACAACGCCCTCAGCATCACC Primer-R:AGCCGCTGGCCCATTCGC TCCAGCGCT	Musa, [13]
4	*SHV*	Primer-F: ATGCGTTATATTCGCCTGTG Primer-R: TGCTTTGTTATTCGGGCCAA	Montso *et al*. [14]
5	*CTX-M*	Primer-F: CGCTTTGCGATGTGCAG Primer-R: ACCGCGATATCGTTGGT	Ali *et al.* [15]
6	*TEM*	Primer-F: AAACGCTGGTGAAAGTA Primer-R: AGCGATCTGTCTAT	Montso *et al*. [15]

Polymerase chain reaction (PCR) is a technique used to amplify a specific DNA sequence. The process begins with an initial denaturation step at 95 degrees Celsius for 10 minutes to denature all double-stranded DNA molecules. This is followed by cycles of thermal cycling, where the denatured strands are separated at 95 degrees Celsius for 1 minute, followed by annealing at a specific temperature for each primer set (58 degrees Celsius for 16S rRNA, 61 degrees Celsius for algD, 65 degrees Celsius for ToxA, 55 degrees Celsius for SHV and CTX-M, and 50 degrees Celsius for TEM) for 1 minute, and then extension at 72 degrees Celsius for 1 minute. The PCR cycle is completed with a final extension at 72 degrees Celsius for 5

minutes. 35 cycles of thermal cycling are performed for each primer set.

The primer sets used in this protocol target the following genes: 16S rRNA for bacterial identification, and algD, ToxA, SHV, CTX-M, and TEM for the detection of antibiotic resistance in Pseudomonas aeruginosa.

Results

The provided susceptibility profile in (table 2) indicates a concerning picture of high antimicrobial resistance amongst the tested bacterial isolates. While Imipenem shows the best performance with 50% sensitivity, all other antibiotics except Tetracycline (no sensitive isolates) exhibit significant resistance: Meropenem at 41.66%, Streptomycin at 20.83%, Amikacin at 25%, Aztreonam at 25%, Levofloxacin at 20.83%, Norfloxacin at 25%, Gentamycin at 20.83%, Ciprofloxacin at 29.17%, Amoxicillin at 8.33%, and Sulfamethoxazole-Trimethoprim at 12.5%. The situation is even worse for Ampicillin and Amoxicillin, showing 100% and 83.33% resistance, respectively. This highlights the urgent need for effective antibiotic stewardship strategies and development of novel agents to combat increasingly resistant bacterial strains. [Table 2].

TABLE 2. Results of antibiotic sensitivity test on isolated *P. aeruginosa*

Antibiotic	Concentration Mcg/ disk	Isolates of *P. aeruginosa* [24 isolates]		
		Sensitive	Intermediate	Resistance
Imipenem [IPE]	10	12 [50%]	9 [37.5%]	3 [12.5%]
Meropenem [MEM]	10	10 [41.66%]	9 [37.5%]	5 [20.83%]
Streptomycin [S]	10	5 [20.83]	12 [50%]	7 [29.17%]
Amikacin [AK]	10	6 [25%]	11 [45.83%]	7 [29.17%]
Aztreonam [ATM]	30	6 [25%]	10 [41.66%]	8 [33.33%]
Levofloxacin [LEV]	5	5 [20.83]	8 [33.33%]	11 [45.83%]
Norfloxacin [NOR]	10	6 [25%]	7 [29.17%]	11 [45.83%]
Gentamycin [GM]	10	5 [20.83]	6 [25%]	13 [54.17%]
Ciprofloxacin CIP	5	7 [29.17%]	4 [16.66]	13 [54.17%]
Tetracycline [TE]	10	0 [0.0%]	8 [33.33%]	16 [66.66%]
Amoxicillin [AX]	25	2 [8.33%]	2 [8.33%]	20 [83.33%]
Sulfamethoxazole-Trimethoprim [SXT]	25	3[12.5%]	0 [0.0%]	21[87.5%%]
Ampicillin [AM]	10	0 [0.0%]	0 [0.0%]	24 [100%]

The NanoPhotometer® is a spectrophotometer that can be used to measure the concentration and purity of nucleic acid samples.

The reading in the figure one shows that the DNA sample has a concentration of 241.6 ng/µl and an A260/A280 ratio of 2.087. The A260/A280 ratio is a measure of the purity of a DNA sample. A ratio of 1.8-2.0 is considered to be pure. The ratio in the figure is slightly higher than this, but it is still within an acceptable range.

The reading also shows the absorbance spectrum of the DNA sample. The absorbance peak at 260 nm is due to the DNA bases, and the absorbance peak at 280 nm is due to protein contamination. The fact that the peak at 260 nm is much higher than the peak at 280 nm suggests that the DNA sample is relatively pure [Fig.1].

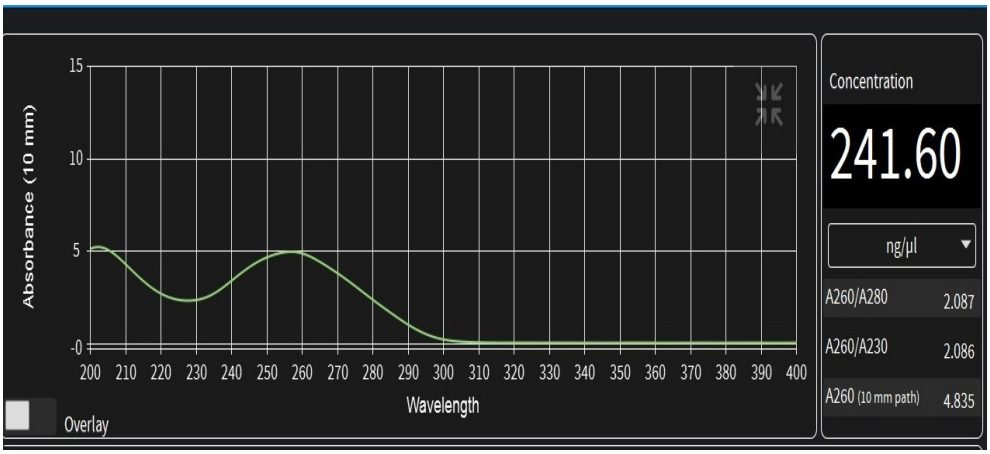

Fig. 1. An example of Implen NanoPhotometer® reading of DNA concentration and purity

The molecular confirmation disclosed that all 24 isolates were *P. aeruginosa* [Figure 2] and came by the results of the gen detection. The outcomes of molecular recognition of virulence genes displayed that 83.33% [20/24] of isolates were positive for the *algD* gene, in contrast, 70.83% [17/24] of them proprietor the *toxA* gene [Fig.2].

The figure 2 suggests that the PCR experiment was successful in amplifying the 16S rRNA, toxA, and algD genes from the isolated Pseudomonas aeruginosa. This information can be used to identify the bacteria and determine if it carries genes associated with virulence or antibiotic resistance.

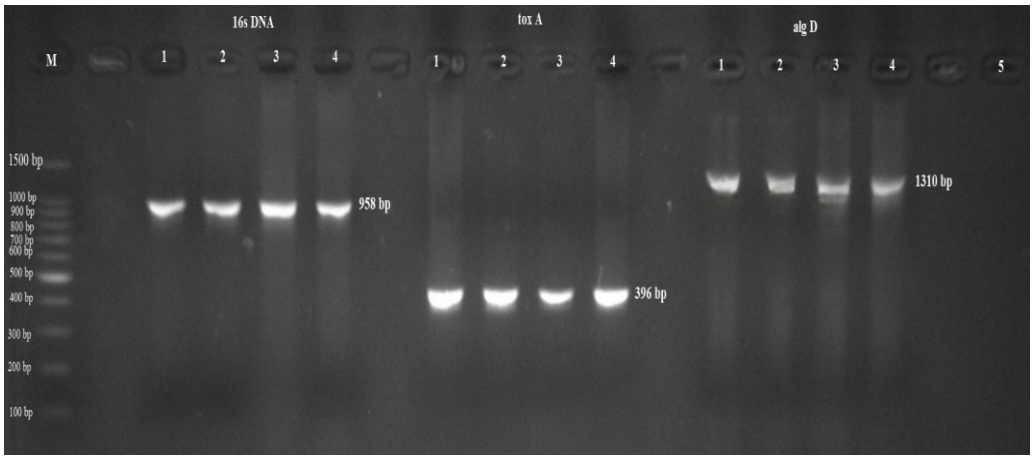

Fig. 2. Amplification of *16S DNA*, *tox A*, and *alg D* genes of isolated *P. aeruginosa*

In figure 3 At least one of the isolates (potentially both in lanes 2 and 3) harbors the TEM gene, suggesting resistance to β-lactam antibiotics like ampicillin and ticarcillin. The faint CTX-M band may indicate weak amplification or low abundance of the CTX-M gene in the isolates. Further investigation might be needed to confirm CTX-M presence and its specific type. The absence of a visible SHV band suggests these isolates likely do not possess the SHV gene, and therefore may not be resistant to extended-spectrum cephalosporins like cefotaxime and ceftriaxone**.**

According to the outcomes of the molecular unearthing of antibiotics resistance genes, the overwhelming majority of isolates carried the CTX-M gene [91.66%], and 75% of them were bearer SHV gene. In comparison, the TEM gene appeared in 45.83% of isolated *P. aeruginosa* [Fig.3].

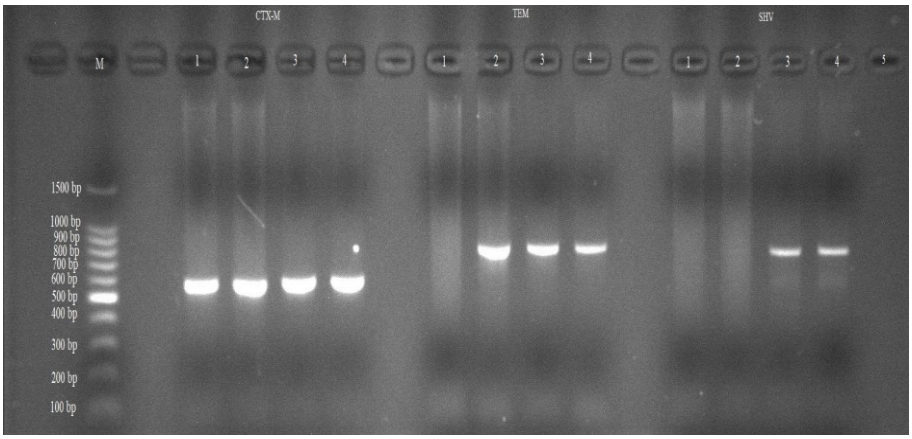

Fig. 3. Amplification of CTX-M, TEM, and SHV genes of isolated *P. aeruginosa*
M: 100 bp marker, 1-4: *Pseudomonas aeruginosa* isolate, 5: control negative

Discussion

One of the primary processes by which organisms adapt to their surroundings is gene expression. In the case of *Pseudomonas aeruginosa*, gene expression influences numerous biological aspects of the bacteria, including its capacity to resist drugs, induce infection, and live in severe settings. There are several ways to study *Pseudomonas aeruginosa* expression of the genes.

Polymerase chain reaction [PCR] is a DNA reaction technology using to greatly increasing the target quantity.

The ToxA gene encodes exotoxin A, a type II extracellular enzyme.This enzyme, alone or in conjunction with other hydrolases, causes acute cell death in the human host, as well as tissue damage and necrosis [17]. ExotoxinA is an ADP-ribosyl transferase that suppresses protein synthesis in human cells by binding to elongation factor 2 [18].

This exoenzyme A is involved in the pathogenicity of *Pseudomonas aeruginosa* in catheter-induced urinary tract infection [19, 20].

In a single study, the ToxA gene was detected in more than 80% of urine isolates [17]. Using a polymerase chain reaction to detect *Pseudomonas aeruginosa* by amplifying the toxin gene, they discovered that 96% of all *Pseudomonas aeruginosa* isolates tested positive [21-23]. Exopolysaccharide Alginate *Pseudomonas aeruginosa* mucoid is distinguished by increased alginate production and decreased motility [e.g., lack of flagellin expression]. The spores protect against changing environmental conditions and promote Adhesion to hard surfaces [24, 25], causing excessive formation of *Pseudomonas aeruginosa* in the AlgD strain of the primary infection [26, 27]. Because of *Pseudomonas aeruginosa's* capacity to avoid the immune system and increase antibiotic resistance [MDR] due to alginate overproduction and eventual biofilm improvement [28].

Pseudomonas aeruginosa is sensitive to the antibiotics imipenem, aminoglycosides, carbapenems, chloramphenicol, and tetracyclines, according to the current study. *Pseudomonas aeruginosa* is also sensitive to the medications Imipenem [IPE] and, Meropenem [MEM] according to the table.

According to the present findings, *Pseudomonas aeruginosa* bacteria can continue to thrive and reproduce in the presence of antibiotics. This can make treating *Pseudomonas aeruginosa* infections difficult.

Pseudomonas aeruginosa resistance is classified into two types: intrinsic resistance, also known as natural resistance or true resistance, which results from genetic traits in bacteria that prevent the effects of antibiotics, and acquired resistance, which is dangerous because it has become more common in recent years [28].

According to [29], this category emerges as a result of isolates acquiring plasmids or jumping factors, while genetic changes can also cause it [30]. Farther the presence of a large number of bacterial strains that are resistant to the Efflux pump system acts as a barrier to the entry of antibiotics into the germs [31]. The bacteria change the target site where the antibody is activated [32]. the presence of enzymes that degrade or change the basic amino acid and One of the primary causes of the emergence of these resistant strains is the overuse and misuse of antibiotics, which results in bacterial resistance to antibiotics[33].

While meropenem and imipenem have shown significant sensitivity, carbapenems are among the most important antibiotics used in the experimental medication for *Pseudomonas infections*. One of the most prominent causes of *Pseudomonas aeruginosa antibiotic resistanc*e is OprD deficiency or a

mutation in the gene associated with the Carbapenem family of medicines.

According to [WHO] [34], carbapenem-resistant *Pseudomonas aeruginosa* is 1 of 3 bacterial species for which new medications to treat infections are urgently needed [35].

Excessive antibiotic use through cure promotes the establishment of multidrug-resistant *Pseudomonas aeruginosa* strains, rendering empiric antibiotic therapy against MRSA ineffective, Antimicrobial resistance is on the rise, posing a hazard to global public health [36].

The risk is heightened by the fact that each gene on the R plasmid, which can carry several genes, each of which encodes resistance to a different class of antibiotic, encodes resistance to multiple antibiotics, making it impossible to battle bacteria with more resistance genes. Furthermore, while resistance is seldom conveyed via transmission and is usually transferred from one bacteria to another, when it does occur, the danger is raised. According to a 2019 research from the Canadian Council of Academies, 26% of bacterial infections in 2018 were resistant to frequently used medicines. However, by 2050, this ratio is predicted to reach 40% [37].

Antibiotics must affect the structure of bacteria in order to eradicate germs through various processes and approaches.

Conclusion

In conclusion, *P. aeruginosa* is the principal bacterial cause of UTI in Humans and has the genetic weapons for considering and persisting infections. In addition, these bacteria own arsenals of antibiotic-resistance genes that construct multi-drug resistant bacteria, which makes the cure and control of infection very hard.

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Author`s contributions

The research is part of a master's thesis study in which the first-named student contributed to conducting the practical part of the study, while the second and third researchers are the supervisors who proposed the topic of the study and contributed to making scientific, linguistic, and structural modifications to the research.

Ethical approve:

Ethical approval bears the number No. 7/18/44/46 on 3/7/2022 according to the instructions of the Ministry of Higher Education and Scientific Research in Iraq.

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التشخيص الجزيئي لبعض جينات الفوعة ومقاومة المضادات الحيوية في جراثيم الزائفة الزنجارية *Pseudomonas aeruginosa* المعزولة من اصابات المجاري البولية

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[2] فرع الأحياء المجهرية - كلية الطب البيطري - جامعة الموصل - الموصل - العراق.
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تهدف الدراسة إلى عزل Pseudomonas aeruginosa من التهابات المسالك البولية باستخدام تفاعل البوليميراز المتسلسل وجين الفوعة والكشف عن مقاومة المضادات الحيوية باستخدام الطرق التقليدية والجزيئية. تم تشخيص جميع عينات UIT للكشف عن P. aeruginosa باستخدام تقنية VITEK-2 Compact التقليدية والكيميائية الحيوية؛ تمت زراعة كل عزلة على طبقتين من أجار ضخ القلب والدماغ، استخدمت إحداهما كمزرعة مخزون لاختبار الحساسية للمضادات الحيوية، بينما استخدمت الأخرى لاستخلاص المواد الوراثية وتضخيمها للكشف عن وجود أو عدم وجود جينات الضراوة والجينات. المسؤولة عن مقاومة المضادات الحيوية. تمت دراسة اختبار الحساسية الدوائية لـ 24 عزلة من بكتيريا Pseudomonas aeruginosa بواسطة طريقة الانتشار القرصي الكلاسيكية ضد [12] المضادات الحيوية على أجار مولر-هينتون. وتراوحت النسبة بين [12.5%] إيميبينيم و[100%] أمبيسلين. أظهر التأكيد الجزيئي أن جميع العزلات الـ 24 كانت من نوع P. aeruginosa وجاءت بنتائج تقنية VITEK-2 Compact. أظهرت نتائج الكشف الجزيئي لجينات الفوعة أن 83.33% [24/20] من العزلات كانت إيجابية لوجود جين algD، في المقابل 70.83% [17/24] منها جين toxA المالك. وفقاً لنتائج التحديد الجزيئي لجينات مقاومة المضادات الحيوية، فإن الغالبية العظمى من العزلات كانت تحمل جين CTX-M [91.66%]، و75% منها كانت حاملة لجين SHV. بالمقارنة، ظهر جين TEM في 45.83% من P. aeruginosa المعزولة. في الختام، تمتلك P. aeruginosa الأسلحة الجينية للتعامل مع العدوى واستمرارها، حيث تمتلك البكتيريا جينات مقاومة للمضادات الحيوية والتي تقوم ببناء بكتيريا مقاومة مما يجعل علاج العدوى والسيطرة عليها أمرًا صعبًا للغاية.

الكلمات المفتاحية: التعريف الجزيئي، المضادات الحيوية، جينات المقاومة، الزائفة الزنجارية.