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Molecular detection of some Vancomycin Pathogenic bacteria in Basrah Governorate

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ABSTRACT

The current study aimed to study the resistance of some species Bacterial resistance to the antibiotic vancomycin. 121 samples were collected and grown on Chocolate Agar medium for isolating pathogenic bacteria. and then It was grown on Nutrient Agar medium for pure colonies. These bacteria were diagnosed using phenotypic methods, biochemical tests, and 16SrRNA gene. The results reported that 40 (33.3%) resistant strains appeared, of which 13 were males with a percentage of (32.5%), while the number of female samples was 27 with a percentage (67.5%) and the rest were sensitive to this antibiotic. done during the study The current study obtained four types of bacteria resistant to vancomycin: 24 strains (60%) belonging to Pseudomonas aeruginosa bacteria, 11 strains (27.5%) belonging to Proteus mirabilis bacteria, 4 strains (10%) of Staphylococcus aureus bacteria, and one strain (2.5%) for Actinomyces sp. As for the presence of resistance genes, Vancomycin, where the current study recorded the presence of 17 bacterial strains containing both VanA and VanB genes (42.5%), while the VanA gene was present in 8 strains (20%), and the VanB gene was present in 6 strains (15%), and 9 appeared Strains (22.5%) do not possess either of the two genes (VanA and VanB), although they were resistant to vancomycin, and this may be due to their possession of other resistance genes or other mechanisms of resistance to this antibiotic.

1. Introduction

Antibiotics are medicines used to treat or prevent a bacterial infection. They either kill or inhibit the growth of bacteria [1]. The modern era of antibiotics began with the discovery of penicillin by Alexander Fleming in 1928[2]. Antibiotics of resistance is defined as the ability of microorganisms to withstand the effect of an antibiotic. In the thirties, the scientist Flemck noticed the emergence of forms of resistance and confirmed this with the emergence of bacteria resistant to penicillin five years after the description of this antibiotic, and this phenomenon was not widespread and did not cause concern for specialists, but it is now a major health problem facing the world. Antibiotic resistance can be either natural or acquired, and natural resistance genes are usually carried on chromosomes, which are passed on to subsequent strains during bacterial division. While acquired resistance results from changes in genetic content (mutation in a

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chromosomal gene or acquisition of a new gene carried on the conjugate plasmid), acquired resistance may occur as a result of resistance genes that have been added to the chromosome through the process of transformation or conjugation process [3]. The World Health Organization has described antibiotic resistance as one of the three most important global health threats in the twenty-first century [4].

Vancomycin belongs to the group of Glycopeptides and was used in treatment in 1950. Its effect is fatal as it inhibits the building of peptidoglycan in the bacterial wall through the process of Transpeptidation [5]. This antibody was isolated from the filamentous bacterium *Streptomyces orientalis*. It is effective against most gram-positive bacteria, including methicillin-resistant S.aureus, *Enterococcus faecalis*, *S. epidermises*, *Clostridium difficile*, and gram-negative spheroid bacteria. The effectiveness of vancomycin against bacteria through its binding to the terminal D-alanyl-D-alanine group depends on the peptide side chain when building the peptidoglycan layer in the cell wall, The complex consisting of vancomycin and D-alanyl-D-alanine blocks the enzyme Transpeptidases that are involved in the synthesis of peptides and disaccharides within the developing peptidoglycan chain, in addition to the enzymes DD-Transpeptidases and D,D-Carboxy peptidases (inhibiting the Transglycosylation process), thus preventing vancomycin from exploiting the substance (substrate) by the enzyme rather than directly interfering with the target enzyme and this is an unusual method of inhibition. Vancomycin-resistant bacteria replace terminal D-alanine with α-hydroxy acid and D-, ie, D-alanyl-D-alanine is replaced by D-alanyl-D-lactate by *VanA* and *VanB* enzymes. Bacteria need five genes that encode five enzymes in order to cause resistance to vancomycin: the enzymes VanH, VanA, VanX, VanY and VanZ [6]. The current study aimed to investigate the pathogenic bacteria that are resistant to the antibiotic Vancomycin, isolate and characterize these bacteria by traditional methods and by amplifying the *16SrRNA* gene and the vancomycin resistance genes (*VanA* and *VanB*).

1. Materials and working methods:

2.1. Sample collection:

121 samples were collected from patients for bacteriological analysis in an appropriate manner to avoid any contamination possible, as 100 samples were collected from Abu Sakhir Primary Health Care Center from two sources: urine and excretion, for both sexes, males and females, and at different ages ranged between (6-65) years, and the collection lasted for a period (6-16/12/2020), and 21 other samples were randomly collected at different ages from Al-Sadr Teaching Hospital in Basra Governorate. The bacteria swabs were grown on chocolate agar medium, and incubated in the Incubator (Binder, Germany) at 37ºC for 24 hours.

2.2. The gDNA extraction:

The gDNA for each isolate was extracted using the kit supplied by Geneaid according to the manufacturer's instructions. The gDNA was detection by electrophoresis on 1% agarose gel which examined by Gel Documentary System.

2.3. Polymerase Chain Reaction (PCR):

PCR technique was used to amplify the diagnostic *16SrRNA* gene for bacterium DNA, as well as this technique was used to diagnose the genes of resistance to vancomycin, *VanA* and *VanB* prefixes shown in Table 1 [7] [8]. Polymerase chain reaction (PCR) was performed at a volume of 50µl according to the leaflet attached with the kit supplied by the manufacturer (Promega) as shown in Table 2. The PCR reaction program was conducted according to the steps as shown in Table 3 and 4 [7].

Table1: The sequence of primers used and the expected gene size

Gene		Primer Sequences	Tm	Gene size	Ta
16S rRNA	R 27	5- AGA GTT TGA TCC TGG CTC AG – 3	60	1550 bp	55
	F 1492	5- GGT TAC CTT GTT ACG ACT T – 3	54		
Van A	F	5- GGG AAA ACG ACA ATT GC-3	50	732 bp	59
	R	5- GTA CAA TGC GGC CGT TA-3	52.4		
Van B	F	5- ATG GGA AGC CGA TAG TC-3	52.4	635 bp	59
	R	5- GAT TTC GTT CCT CGA CC-3	52.4		

Tm: Melting temperature Ta: Annealing temperature F: Forward R: Reverse

Table 2: Multiplex PCR reaction mixture components.

Chemicals	Volume (µl)
Master mix (Promega)	25
Forward Primer, *VanA* (10 pmol/µl)	2
Reverse Primer, *VanA* (10 pmol/µl)	2
Forward Primer, *VanB* (10 pmol/µl)	2
Reverse Primer, *VanB* (10 pmol/µl)	2
Template DNA	5
Nuclease free water	12
Total Vol.	50

Table 3: *16SrRNA* gene amplification program

Stage	Steps	Temperature	Time (min)	Cycle number
First	Initial denaturation	95^0C	5 min	1
Second	Denaturation	95 ^{0}C	30 sec	35
	Annealing	55 ^{0}C	30 sec	
	Extension	72 ^{0}C	30 sec	
Third	Final extension	72 ^{0}C	5 min	1

Table 4: *VanA* and *VanB* genes amplification program.

Stage	Steps	Temperature	Time (min)	Cycle number
First	Initial denaturation	95 ^{0}C	5 min	1
Second	Denaturation	95 ^{0}C	30 sec	40
	Annealing	59 ^{0}C	30 sec	
	Extension	72 ^{0}C	30 sec	
Third	Final extension	72 ^{0}C	5 min	1

2.4. Detection of PCR products by electrophoresis:

After the time of the amplification program for the *16SrRNA* gene amplification products, as well as the same process after the amplification of the vancomycin-resistant (*VanA* + *VanB*) genes, the electrophoresis was carried out on 2% agarose gel, put in the first hole 5 microliters of DNA ladder with a size of 2000 bp, and in the other holes 10µl of PCR amplification products were added and the voltage was set at 70 volts for 45 minutes and after the migration process was completed the template was transferred and the gel was examined by Ultraviolet (UV- ray) Gel documentation System, and then recorded the results after photographing them directly by the camera.

3. Results:

3.1. Molecular study:

Genomic DNA was extracted from bacteria using the equipment supplied by Geneaid Company, and the extracted DNA from the different bacterial isolates was detected by electrophoresis on 1% agarose gel containing Ethidium bromide dye at a voltage of 70 volts as shown in Fig. 1.

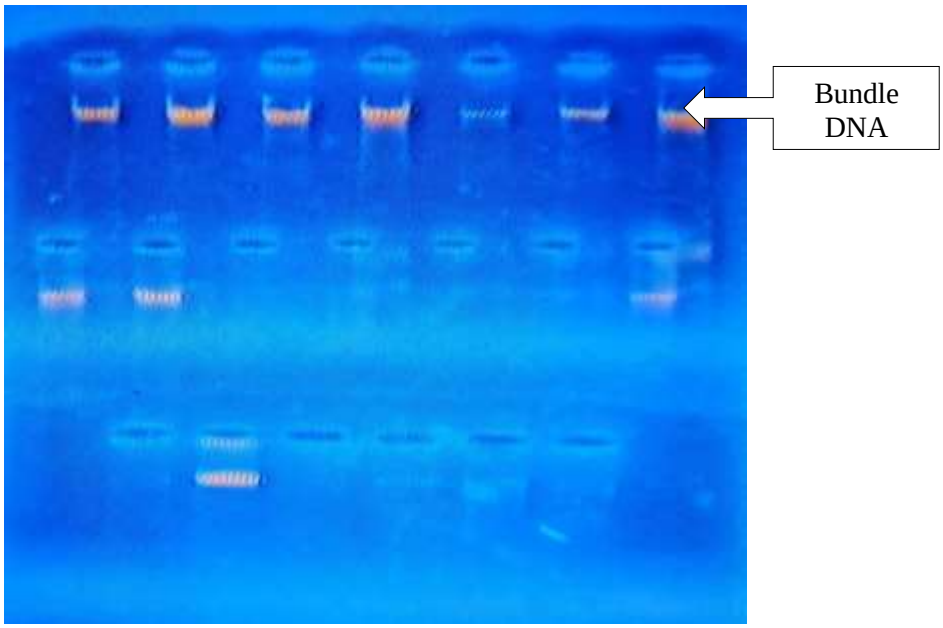

Fig. 1. Genomic nucleic acid DNA bundles during migration electrophoresis on a 1% agarose gel.

3.2. Bacterial diagnosis by *16SrRNA* gene :

The results of amplification of the *16SrRNA* gene were shown for the isolated samples, as the results were positive and the gene size was 1550 bp with a 45-minute relay at 70 volts (Fig.2).

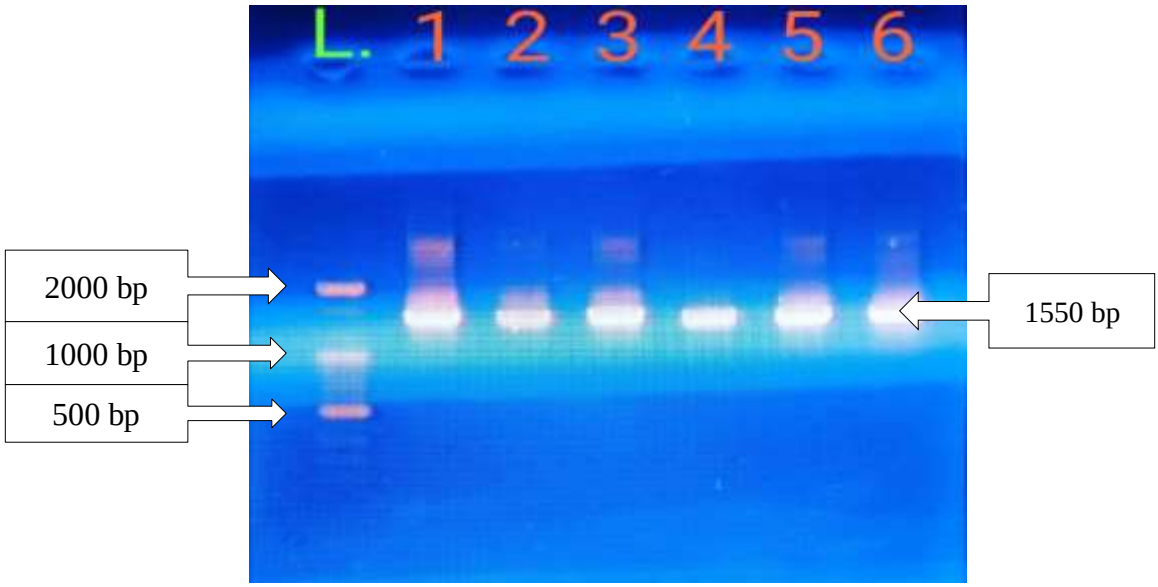

Fig.2. Electrophoresis of *16SrRNA* gene PCR products on 2% agarose gel, M = Marker (100-2000 bp).

3.3. Detection of Vancomycin resistance genes by PCR :

The results of amplification of the *VanA* and *VanB* genes of the isolated samples showed that the result was positive, and the *VanA* gene was 732 bp in size, and the *VanB* gene was in the size of 500 - 635 bp, for 45 minutes and at an amount of 75 volts, as shown in Fig. 3.

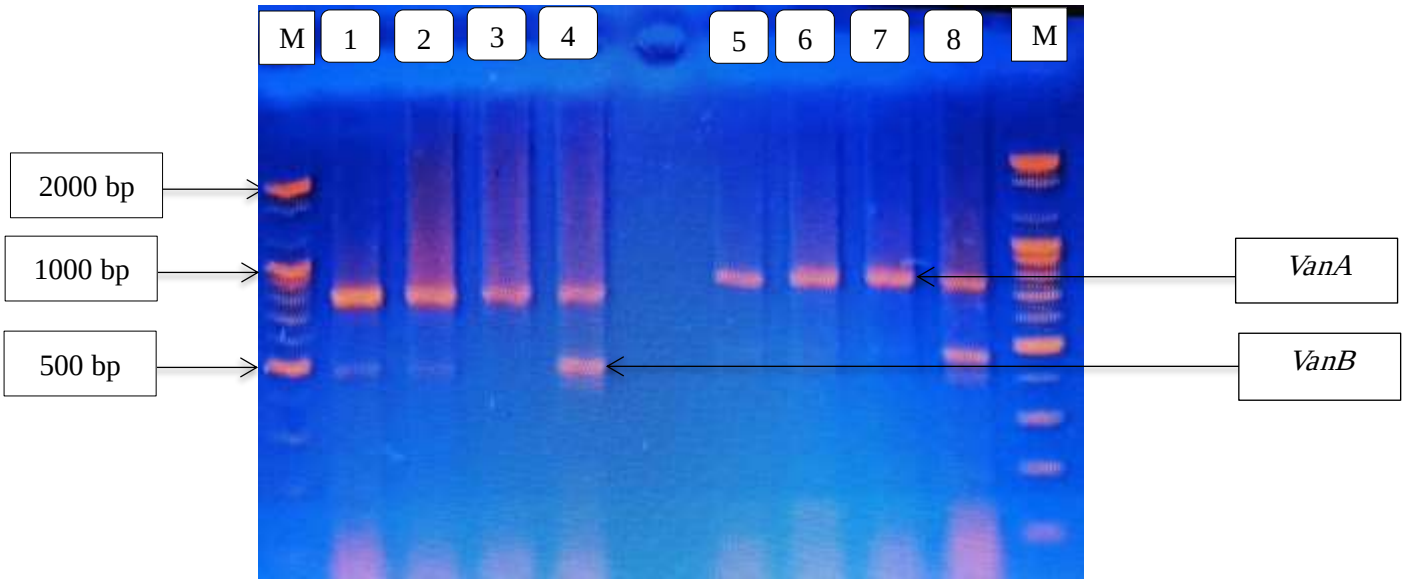

Fig. 3.Electrophoresis of *VanA* and *VanB* genes PCR products on 2% agarose gel, M = Marker (100-2000 bp).

The results of the current study showed that there are isolates possessing the resistance gene *VanA* only, some possessing the *VanB* gene only, and some possessing both the *VanA* and *VanB* genes, and isolates did not possess either of them as in Table (5) and Table (6).

Table 5: Percentages of bacterial isolates containing vancomycin resistance genes.

Total number of isolates resistant to Vancomycin	VanA gene	VanB gene	VanA and VanB genes	Do not VanA and VanB Genes
40	8	6	17	9
Percentage	20%	15%	42.5%	22.5%

Table 6: The number and presence of plasmid resistance genes and their proportions in the studied bacterial isolates.

No.	Isolates	*VanA* gene	*VanB* gene	*VanA* and *VanB* genes	Do not *VanA* and *VanB* genes
1	*Pseudomonas aeruginosa*	5 (62.5%)	2 (33.3%)	14 (82.4%)	3 (33.3%)
2	*Proteus mirabilis*	3 (37.5%)	2 (33.3%)	2 (11.8%)	4 (44.4%)
3	*Staphylococcus aureus*	0 (0%)	1 (16.7%)	1 (5.9%)	2 (22.2%)
4	*Actinomyces* sp.	0 (0%)	1 (16.7%)	0 (0%)	0 (0%)
	Total	8 (20%)	6 (15%)	17 (42.5%)	9 (22.5%)

4. Discussion:

The widespread use of glycopeptide antibiotics, including vancomycin, has led to the emergence of many strains resistant to this antibiotic. Infection with vancomycin-resistant bacteria in their ability to acquire genetic factors that may encode resistance to other antibiotics, and these factors may be exchanged with other species, which constitutes one of the main health problems, and therefore its spread will reduce the possible treatment options for doctors [9].

The results of the current study showed that 8 isolates (20%) possessed the *VanA* gene, 6 isolates (15%) possessed the *VanB* gene, and (17) isolates (42.5%) possessed both *VanA* and *VanB* genes, while the rest of the isolates (9) did not. Either of the two genes appears in it, although it was also resistant to vancomycin, and this may be due to having another non-genetic resistance mechanism or to having other resistance genes. The *VanA* gene bundles were 732 bp in size, and the *VanB* gene bundles were 500-635 bp in size. In general, there was a relative dominance of the *VanA* gene over the *VanB* gene.

The results of the current study were similar to the study of [10], who identified 9 isolates (1.1%) as vancomycin-resistant Enterococci (VRE), two of them had the *VanA* gene, one wasolate had the *VanB* gene, and six wasolates had The *VanC* gene. [11] conducted a study of the genetic content of isolates of *Staphylococcus* spp. And *Enterococcus* spp. Which showed resistance to the anti-vancomycin, and the results of the migration showed that some isolates contained only one plasmid bundle, while the rest of the isolates appeared devoid of plasmid bundles. [12] study showed that the results of investigation of vancomycin resistance genes in clinical and environmental isolates of *Staphylococcus* spp. One clinical isolate CSb68 possessed the *VanA* gene, while for the *VanB* gene, three isolates showed the *VanB* gene, and this converges with the results of the current study.

The results of the investigation of vancomycin resistance genes in Vancomycin-resistant-enterococci (VRE) bacteria in the study of [13] between E.faecium and E.faecalis showed that *VanA* resistance genes are more prevalent among local isolates compared to *VanB* genes. [14] showed that the majority (84%) of the Vancomycin-resistant Enterococcus faecium isolates possessed the *VanA* gene, and the rest of the isolates (16%) were Enterococcus faecalis bacteria that possessed the *VanB* gene. [15] study conducted PCR reaction test for chromosomal and plasmid DNA using the *VanA*, *VanB*, and *VanC* primers for 31 isolates of *E.coli* resistant to vancomycin. In comparison with the study of [16] out of 129 enterococci isolates that were 56 strains (43.4%) resistant to vancomycin, the *VanA* gene was detected in 54 strains (91.5%), while none of the strains were detected. The strains contained the *VanB* gene.

In the study of [17], the resistance of 100 isolates of S.aureus bacteria to vancomycin and methicillin was tested using PCR technique. These isolates included 54 isolates from animals and 46 isolates from humans. The results of the study showed that there are 49 isolates from animals and 37 isolates from humans. Clear bands with a size of 228 bp were considered to be *Staphylococcus aureus* isolates, of which three isolates were from humans, which showed clear bands with a size of 314 bp. Together. As for the study by [18] on S*taphylococcus aureus*, the *VanA* gene was diagnosed in 18 isolates (17.4%), and the *VanB* gene in 9 isolates (8.7%) of these bacteria resistant to vancomycin, and these two studies are close in their results with the results of the study current.

5. Conclusions:

This study concluded that some types of Gram-negative bacteria were also resistant to vancomycin, and *Pseudomonas aeruginosa* was the most frequent Gram-negative bacteria to vancomycin resistance by 60%. Many species of Gram-negative and positive bacteria were carrying one or both vancomycin resistance genes *VanA* and *VanB*, and resistance may be caused by the acquisition of resistance genes by cross-transmission of those genes.

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التشخيص الجزيئي لجينات مقاومة المضاد الحيوي الفانكومايسين لبعض الانواع البكتيرية في محافظة البصرة

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الكلمات المفتاحية

البكتريا المقاومة للفانكومايسين، جين Van A ، جين Van B ، 16SrRNA

الملخص

هدفت الدراسة الحالية الى دراسة مقاومة بعض الأنواع البكتيرية المقاومة للمضاد الحيوي الفانكومايسين. إذ جمعت 121 عينة، ونميت على وسط Chocolate Agar لغرض عزل تلك البكتريا المرضية. وبعدها نميت على الوسط المغذي Nutrient Agarللحصول على مستعمرات نقية. شخصت تلك البكتريا باستعمال الطرق المظهرية والاختبارات الكيموحيوية وتأكد تشخيصها بالطرق الجزيئية من خلال الجين التشخيصي *16SrRNA* فظهرت 40(33.3%)عزلة مقاومة لهذا المضاد، منها 13 عينة من الذكور وبنسبة (32.5%) بينما بلغ عدد عينات الاناث 27 وبنسبة (67.5%) والمتبقية كانت حساسة لهذا المضاد. تم خلال الدراسة الحالية الحصول على أربعة أنواع بكتيرية مقاومة للفانكومايسين وهي: 24 عزلة وبنسبة (60%) تعود لبكتريا *Pseudomonas aeruginosa* و 11 عزلة وبنسبة (27.5%)تعود لبكتريا *Proteus mirabilis* و 4 عزلة وبنسبة (10%) من بكتريا *Staphylococcus aureus* وعزلة واحدة وبنسبة (2.5%) للبكتريا *Actinomyces* sp. أما بالنسبة لتواجد جينات مقاومة الفانكومايسين حيث سجلت الدراسة الحالية وجود 17 عزلة بكتيرية حاوية على كلا الجينين VanA و VanB وبنسبة (42.5%)، بينما تواجد الجين VanA في 8 عزلات وبنسبة (20%) وجين VanB في 6 عزلات وبنسبة (15%)، كما ظهرت 9 عزلات وبنسبة (22.5%) لا تمتلك أي من الجينين VanA و VanB رغم انها كانت مقاومة للفانكومايسين ولعل هذا يعود لامتلاكها جينات مقاومة أخرى أو أليات أخرى لمقاومة هذا المضاد.

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