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Detection Of Panton-Valentine Leukocidin Gene In Methicillin-Resistant S.Aureus Isolated From Different Sources In Kirkuk-Iraq.

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Abstract

Panton Valentine Leukocidin is a cytotoxin produced by most methicillin-resistant strains in the community. It is encoded by two genes, LukS-PV and LukF-PV. It is transmitted by Bacteriophage, causing lysis of white blood cells and tissue necrosis;when it spreads in the lung, it causes severe pneumonia that leads to death.300 samples were collected including 200 clinical samples that include samples of nose, urine, wounds and burns from different hospitals and centers in Kirkuk. 100 environmental samples include 50 samples of soil and water taken from Kirkuk areas and 50 food samples that include milk, cheese, meat from shops and butchery for the period from 1st November to 20thApril, 2021.60 clinical isolates and 27 environmental isolates were Staphylococcus aureus. They were diagnosed using phenotypic, microscopic, biochemical and confirmatory tests using the Api-Staph system. They were divided into 34 isolates from the nose, 9 from urine, and 8 from wounds and 9 isolates from burns, while the environmental isolates include 11 isolates from soil, 5 isolates from water, 5 isolates from milk, 4 isolates from meat and 2 isolates from cheese. Methicillin-resistant strains were detecte based on both of Oxacillin and Cefoxitin. It was found that 32 of 60 clinical isolates and 13 of 27 environmental isolates were MRSA.A sensitivity test was conducted for 15 common antibiotics.The bacteria showed a high sensitivity to Naldexic Acid by 71.1%,while all isolates were resistant to Oxacillin, Amoxicillin, Cefoxitin, and Vancomycin by 100%.The gene encoding PVL was detected in all MRSA isolates using Conventional PCR technique, where the results showed the presence of the gene in 30 clinical isolates with a percentage of 94.17%,including 15 isolates from the nose, 5 isolates from urine,5 isolates from burns and 5 isolates from wounds. As for the 13 environmental isolates, the percentage of gene presence in them was estimated at 83.2%, they included 5 isolates from soil, one isolates from water, and two isolates Of meat, one isolate of cheese and one isolate of milk

Introduction

Staphylococcus aureus are spherical Gram-positive cells, usually arranged in irregular clusters resembling a grape cluster, or they may be single-celled, pairs, tetrads, or short chains. positive for catalase and coagulase and negative for oxidase.It is considered one of the main human pathogens represented by infections acquired from Communityand hospitals. It is present form a normal flora on the surface of the skin and mucous tissues

(1).The severity of infection starts from simple to more serious and may lead to death by 20-30%,because its resistance for many commonly used antibiotics such as Vancomycin, Erythromycin, Cefoxitin, Rifampin and other antibiotics, Methicillin is one of the most antibiotics that bacteria are resistanted at the present time, it is called resistant strains of MRSA (2) and the risk of infection with these strains increases because resistance to beta-lactamases So its ability to produce the enzyme beta-lactamase, as well as it has the virulence factors such as the coagulase enzyme and Hemolysin, DNAase, and the enterotoxins and cytolytic toxins, including the poison that analyzes white blood cells (3)(Michael et al., 2020). PVL is associated with skin infections and soft tissue necrosis, produced by methicillin -susceptible Staphylococcus aureus (MSSA) and methicillin -resistant Staphylococcus aureus (MRSA)(4).This toxin consists of two exogenous proteins encoded by two neighboring genes (lukS-PV and lukF-PV) that destroy white blood cell membranes and soft body tissues, and may cause hemorrhagic pneumonia when infection spreads to the lungs and causes rapid lung tissue destruction and has been proven fatal in 75% of cases (5).The PVL gene can be transmitted between strains by phages and plasmids, and its spread is less common among MSSA strains compared to MRSA strains. There is a large variation in the prevalence of PVL between S. aureus and infections caused by MRSA-resistant strains throughout The world where the prevalence of the gene has been reported at rates ranging from 12 to 90% in different countries such as the United States of America, China, Germany, Egypt, Nigeria, and Kenya. While other studies have shown that the prevalence of the PVL gene in France is 5%, Bangladesh 14.3%, Saudi Arabia 8.1%, United Kingdom 8.1%, Gambia 72.7%, Nigeria 10.7% and Ghana 4.1%(75%)(6). Becausefew of available information on the prevalence of this gene in clinical and environmental isolates in Iraq, this research was .conducted.

2. And Methods Materials

1.2Collection of samples

200clinical samples (nose, urine, wounds, burns) were collected from patients of both sexes from different hospitals and health centers in the city of Kirkuk, and 100 environmental samples included (soil, water, milk, cheese, meat) from different areas and shops in Kirkuk for the period. From 1stNovember to 20thApril, 2021 and transferred to the laboratory

2.2Isolation and Identification

The samples were planted on Mannitol Salt agar medium by the planning method, and the dishes were incubated at a temperature of 37 °C for 24 hours and then diagnosed based on bacteriological phenotypic, microscopic and biochemical tests, by studying the planting characteristics of the colonies growing on the culture media in terms of shape, size and color and distinguishing them if they were fermented for mannitol or not. The Coagulase test was also conducted. The diagnostic biochemical tests included the oxidase test, the catalase test, urease and others. Then the confirmatory tests were conducted using the Api20-Staph system. (7)

3.2 Antibiotic resistance test

The sensitivity of S.aureus to antibiotics was tested using the baure-kirby method as stated in(8)and then the results were compared with standard tables (9).

4.2 Detection methicillin-resistant S.aureus (MRSA)

It includes the use of oxacillin and cefoxitin tablets by the disk diffusion method (10), by inoculating Muller Hinton agar medium with S.aureus by diffusion method, then placing the oxacillin and cefoxitin tablets and incubating the dishes at 37 °C for 24 hours, then The diameters of the damping zone were measured and compared with the standard values mentioned in (9).

5.2 Primer sequence

Gene	Gene Size	(Primer Sequence′5–3′)		Reference
PVL	433	F	5’- ATCATTAGGTAAAATGTCTGCACATGATCCA-3	(11)
		R	5’-GCATCAACTGTATTGGATAGCCAAAAGC-3	

6.2 Steps used to detection PVL gene in S.aureus

1.6.2 Genomic DNA extraction

The kit supplied by Addbio Company was used for the purpose of extracting the genomic DNA, as the extraction steps were carried out according to the attached protocol, and a dropspectrophotometer Nanodrop was used to measure the purity and concentration of the DNA genomic

2.6.2Polymerase chain reaction(PCR)

This reaction mixture was prepared using the AccuPower PreMix® PCR kit and according to the manufacturer's instructions, the polymerase chain reaction mixture was prepared in the PCR tubes equipped with the kit and containing the reaction components with the addition of other components. Then the tubes were closed and mixed well with a Vortex device for five seconds. The tubes were then transferred to the Thermocycler for the purpose of DNA amplification according to the optimum thermal conditions for the cycles, and the device was programmed according to the interaction shown in Table (1-2).(11)

Table 1-2:Optimal conditions for performing PCR

Stage	Temperature	Time	Number of cycle
Initial DNA Denaturation	94	3 min	1cycle

DNA Denaturation	94	45 s	33cycle
Annealing	58.5	45 s	
Extention	72	2 min	
Final Extention	72	10 min	1cycle

3.6.2Detection for the presence of amplified DNA bands

4µl of DNA Ladder (50-1500bp) is transferred to the first hole for amplified DNA volume measurement, then 5 µl of amplified DNA is transferred to pits in 1.5% agarose gel, then the surface of the gel is immersed in 1X TBE buffer, and then migrated The samples were passed over a voltage of 80 volts for an hour and the gel containing the PCR product was examined using a UV-transiluminator with a wavelength of 260 nm

4. Results and Discussion

1.4Isolation and diagnosis

The study showed the presence of bacteria in 60 clinical isolates and 27 environmental isolates, distributed according to the type of isolate, as shown in Figure (1-4), (2-4)

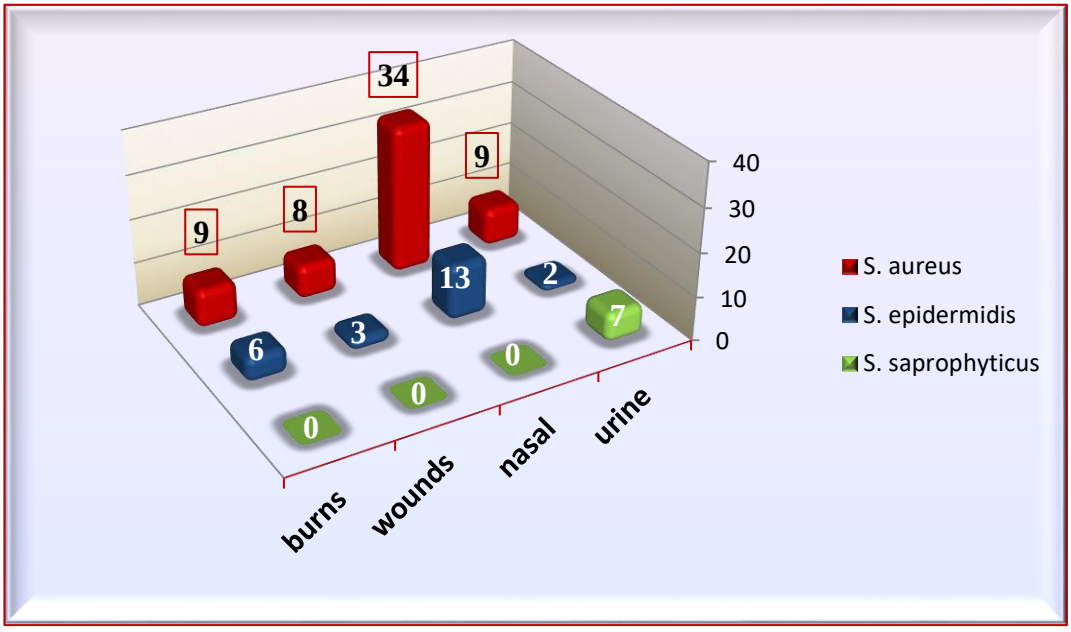


Figure (1-4):Distribution of bacterial species according to the type of clinical sample

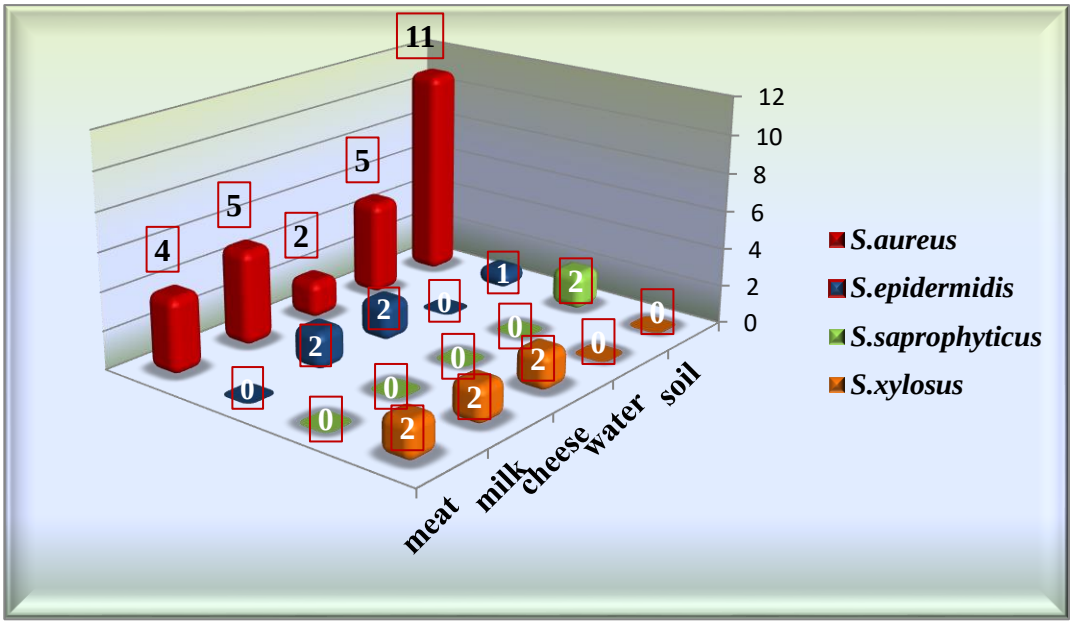


Figure (2-4):Distribution of bacterial species according to the type of environmental sample

2.4 Detection for MRSA and response to antibiotics

The results of the study showed that out of 60 clinical isolates, 32 of them were resistant to methicillin with a rate of 53.3%, which is contrary to what was reached by (12),where the study indicated that the percentage of resistant isolates is 95.5%, and the resistance rate increases over time as a result of the excessive use of antibiotics that lead It led to the production of new strains carrying the mecA gene on plasmids and 27 environmental isolates, 13 of which were resistant with a rate of 48.1% similar to what the researchers reached (13),where he indicated that all of the isolates were tested with an estimated resistance rate of 41.48%. S.aureus isolated from environmental and clinical samples towards 15 antibiotics by disc diffusion method on muller hinton agar medium according to the Organization of Clinical Laboratories Standard (9)to determine the sensitivity or resistance of bacterial isolates to antibiotics. The bacteria showed a variation in their response to commonly used antibiotics. More sensitive to Naldexic Acid by 71.1% as it is one of the antibiotics that has a strong effect on the DNA of bacteria and thus prevents the formation of proteins, while all isolates were resistant Cefoxitin, Vancomycin, Oxacillin, Amoxicillin, due to the bacteria's possession of resistance genes carried on the plasmid as well as the ability to produce the beta-lactamase enzyme that works to break down the beta-lactam ring forming the antibiotic wall or the bacteria works to form a biofilm that increases the thickness of the wall and prevents the antibiotic from Access to it, and the reason for this resistance may be due to the transfer of genes encoding VanA carried on conjugate plasmids or jumper genes from other strainThe total resistance you use against vancomycin, as it converges with the findings of the researchers (14) by 97%, as well asS.aureus showed resistance to most other commonly used antibiotics as shown in Figure(3-4).The reason for the difference in the proportions of the current study with previous studies is due to the different social habits of people and geographical location

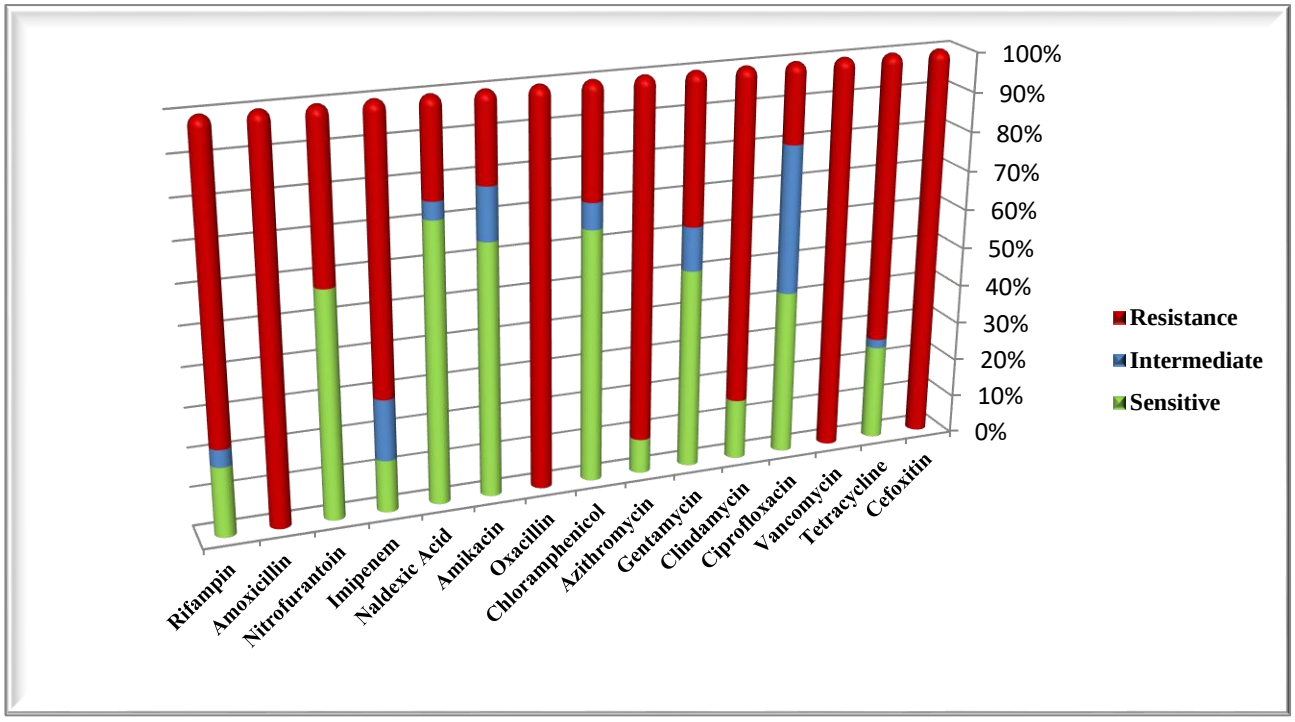


Figure: 3-4 percentages of clinical and environmental samples

5.4 Polymerase Chain Reaction (PCR)

2.5.4Result of the PCR reaction to investigate the PVL gene of the MRSA isolates under study

All clinical and environmental isolates from the MRSA polymerase reaction mixture were tested and distributed by sample type as shown in Figure (4-4) and (5-4) respectively, to confirm the presence of the gene responsible for PVL production

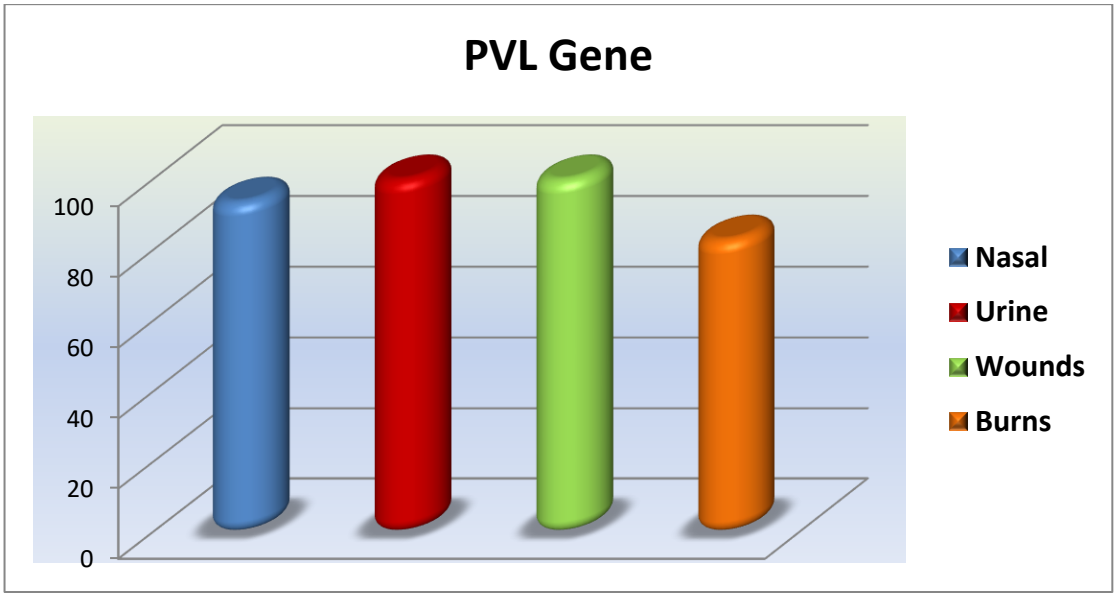
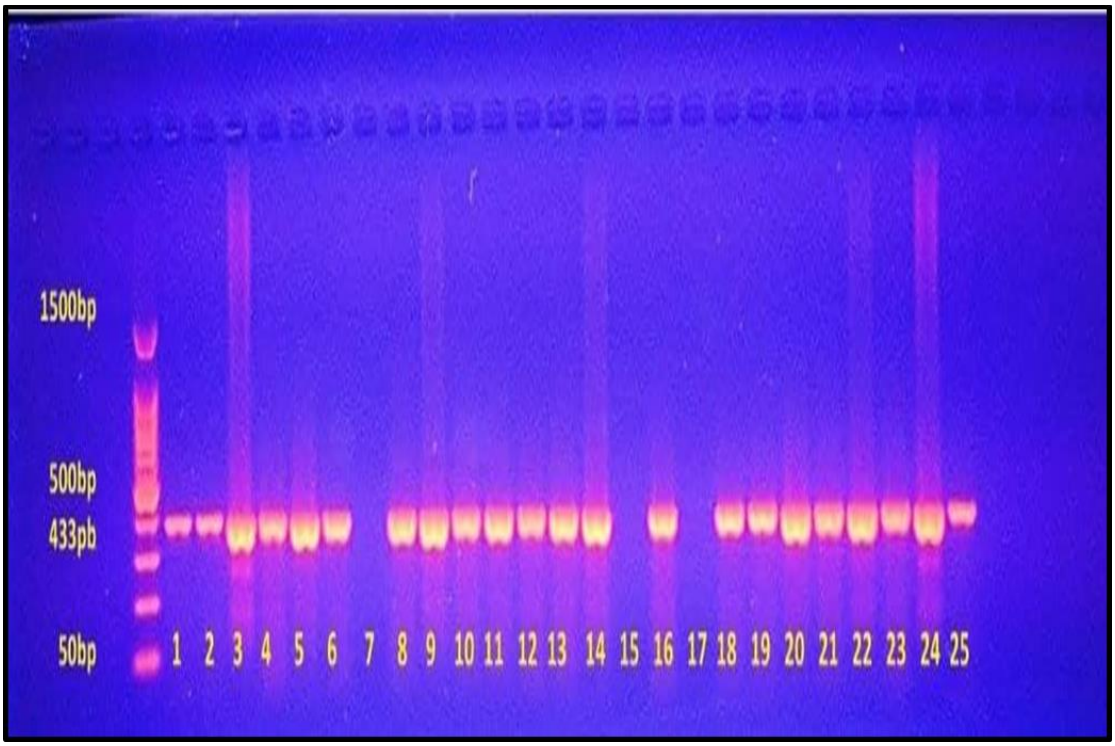
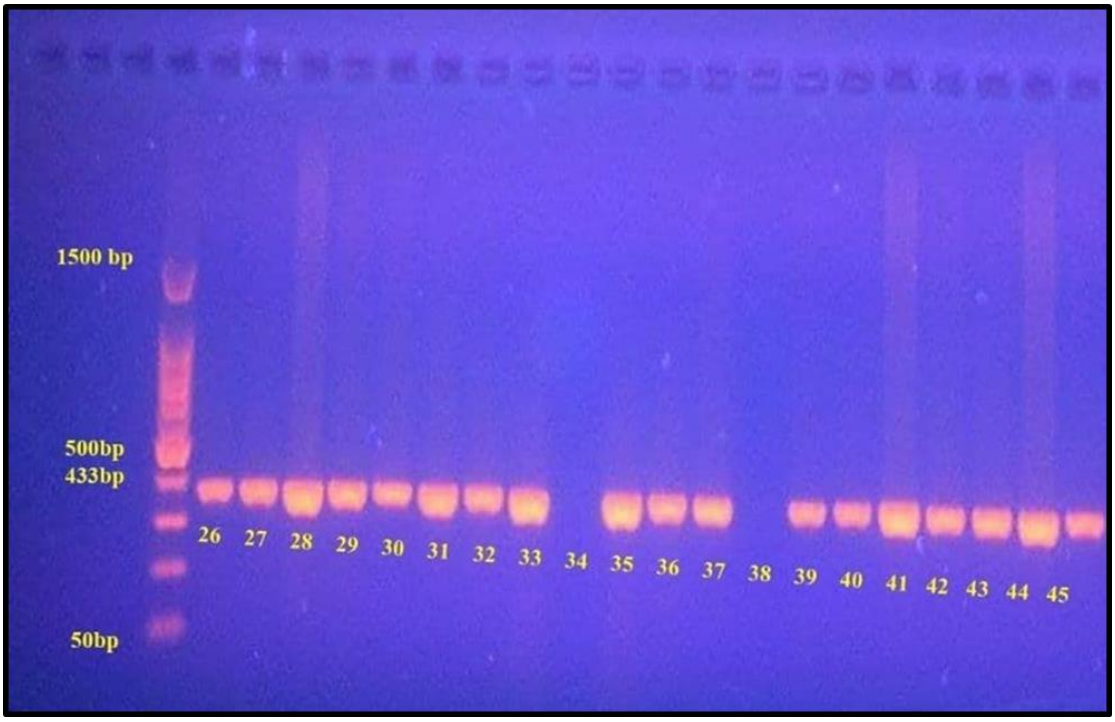


Figure:4-4 shows the percentages ofclinical isolates carrying the PVL gene

The results showed that among 32 clinical isolates resistant to methicillin, 30 of them carried the gene encoding PVL, which is one of the genes found in MRSA isolated from the community. Among 54 clinical isolates, only 14 of them are carriers of the gene. In the current study, the distribution according to the type of clinical samples of the 16 nasal isolates, 15 of them carried the PVL gene with a rate of 93.7%, and this result is close to what was reached by (15),where he indicated that 15 out of 18 isolates were taken from the nose of the workers in Restaurants carry the gene. All isolates of urine and wounds showed that they carried the gene with 100%, and this is in agreement with what was found in the study of(16) which indicated that all 57 isolates carried the gene at 100%. while the burn samples were among 6 isolates, 5 of which were carriers. As a result of direct contact with the surrounding environment, contamination of the skin occurs, thus infection with pathogenic bacteria, while the study conducted by (17) showed that 13 out of 47 clinical isolates of MRSA are carriers of the gene. In pictures (1-4) (2-4)



picture 1-4: Amplification products of the PVL gene (433bp) of MRSA bacteria using electrophoresis PCR technique on agarose gel (1.5%) with a potential difference of 80V (80V) for an hour and a half, where M represents the volume guide (DNA Ladde(50-1500). Nasal isolates 1-14,18,19 ((((15-17)) Milk isolates, (20-24) Diuretic isolates, figure (25) .Cheese isolates



picture 2-4: Supplementary products for amplification of the PVL gene with a molecular size (433bp) of MRSA bacteria using electrophoretic PCR technique on a 1.5% agarose gel with a potential difference of (80V) for an hour and a half, as M is the size guide (50-1500bp)DNA Ladder. The number (30-26) represents isolates of wounds, (36-31) isolates of .burns, (42-37) isolates of soil, (44-43) of meat isolates, and (45) isolates of water

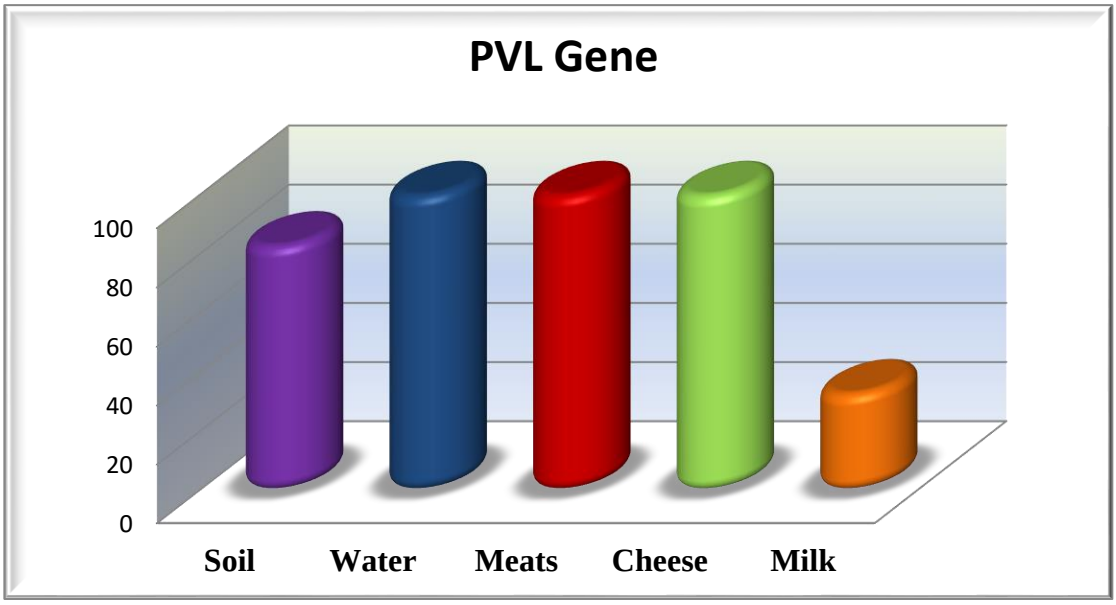


Figure 5-4 shows the percentages of environmental isolates carrying the PVL gene

As for the environmental samples represented by soil, they were among 6 isolates, 5 of which carried the gene of 83%. In the cleanliness of the place, as for the water and cheese isolates, as well as the two meat isolates, they carried the gene by 100%, and among the 3 milk isolates only 1 contained the gene by 33%, and this percentage is close to what was

reached (18),where it showed that the of the 65 isolates that are resistant to methicillin, only 26 are carriers of the gene. The reason for the widespread spread of bacteria in environmental samples, which includes food samples, is the increase in pollution resulting from lack of health awareness in dealing with foodstuffs such as cheese, milk and meat, as the lack of attention to personal hygiene among workers in restaurants causes most of the infections in restaurants. Cases of food poisoning (19). What increases the danger of the presence of these breeds in the environment is that they have evolved to a large degree to withstand the changing environmental conditions of pH and temperature. The percentage of the gene's presence in clinical isolates is estimated at 94.17% and the environmental isolates at 83.2%. The study concluded that the presence of the gene can reliance on a clinical indication of MRSA, but it may not have the ability to express the genes and the evidence for this its presence in environmental isolates at a high rate despite its mechanism of action It is the lysis of white blood cells and tissue necrosis, and this is found in clinical sources only, so it can be confirmed that the source of environmental isolates is mainly clinical as a result of direct and indirect contact between humans and the external environment that causes bacterial infection, as well as what increases the risk is the widespread spread of Bacteriophage in The community where the gene encoding PVL is carried on the phage, which fuses with the chromosome of S.aureus and not degrade it, but rather increases its virulence. The greatest interest is in MRSA, because it's not only resistance to methicillin, but also has the ability to resist all the antibiotics used, that is,it not respond to treatments and therefore weakness In a person's immune system as a result of thedecomposition of white blood cells, and this is one of the main causes of death resulting from infection with methicillin-resistant Staphylococcus aureus strains that contain the gene in communities

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