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Molecular Detection of Virulence Factors Genes for Some Species of Bacteria That Cause Otitis Media in Kirkuk Governorate

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Abstract:

The study included collecting 100 samples from individuals who visited the Department of Ear, Nose, and Throat (ENT) Unit in Azadi Teaching Hospital, Hawija General Hospital, and outpatient clinics in Kirkuk Governorate. The samples were collected from individuals aged 2-45 years, during the period from October 27, 2022, to January 3, 2023. The bacterial culture results revealed that 91 samples, accounting for 91% of the total, exhibited bacterial growth on the Mannitol salt agar, MacConkey agar, and Blood agar media. 9 samples and 9% of the samples did not give bacterial growth The results detection of virulence factors showed the ability of *Staph aureus* to form biofilm and product hemolysin percentages 100% respectively .while *Kocuria kristinae* bacteria formed a biofilm and produced hemolysin, protease, and urease enzymes and beta-lactamase-percentage 100% respectively. While the results showed a decrease in the ability of *Proteus mirabilis* bacteria to form biofilm by a percentage of 60%, Also the ability of *Morganella morganii* bacteria to form a biofilm and produce hemolysin and protease enzymes decreased by percentage 20,40,40% respectively. The results showed the ability of *E.coli* percentage of 75% to form a biofilm and produce a hemolysin percentage of 100%. Only 50% of *Klebsiella pneumoniae* bacteria isolated showed the ability to form a biofilm. While product hemolysin and urease enzymes percentage 75% respectively. PCR was used to determine the presence of (*hlaB*) in *Staph aureus* bacteria, (*ndvB*) in *E. coli*, (*UreR*) in *P. mirabilis*, (*Prot_clp*) in *K. kristinae*, found in, 100%, 50%, 100%, 100%, respectively.

Keywords: Otitis media infection, pathogenic bacteria, virulence factors, specific primers, PCR.

1. Introduction

Otitis medium is a middle ear cavity infection that can be caused by various factors, including bacteria, viruses, or fungi [1]. Bacterial inflammation refers to the presence of bacteria in the body that can lead to disease. This occurs when bacteria enter the body or migrate from their usual location, such as Enterobacteriaceae in the digestive system. This migration can occur due to weakened immunity in the individual or when the bacteria invade other tissues. Skin infections cause numerous infections that spread to other organs and may cause fatal septicemia. The swimming in river water may cause otitis infection because contains many organisms

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such as bacteria, fungi, and parasites [2,3]. Pathogenic bacteria are the most common cause of ear infections.

Exogenous (OE) bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis*, Enterobacter, *Klebsiella pneumoniae*, and *E. coli* followed by fungi such as Aspergillus spp. And *Candida albicans*, and sometimes viruses such as *Herpivirus hominis* and Varicella-zoster virus. [4]. The middle ear is susceptible to infection by the respiratory tract such as bacteria, and viruses, In addition, the presence of the tympanic membrane facilitates to exposure opportunistic pathogens that can cause middle ear infections[5]. Children are more vulnerable to infection than adults [6]. Several studies have revealed that over 80% of youngsters in the United States experience this specific type of inflammation [7]. Timely identification of this illness is crucial, even in the absence of a dependable diagnostic examination during its initial phases. Erroneous diagnosis results in delayed therapy, improper use of treatment, or excessive consumption, all of which heighten the likelihood of complications and foster resistance to Antibiotics [4].

This study aimed to:

a. Isolation and identification of some types of bacteria that cause otitis media in Kirkuk Governorate for age groups ranging from 2-45 years.
b. Molecular detection of genes encoding some of its virulence factors.

1. MATERIALS AND METHODS

1.1. Collection of samples

The study included collecting 100 samples from individuals who visited the Ear, Nose, and Throat (ENT) department at Azadi Teaching Hospital, Hawija General Hospital, and outpatient clinics in Kirkuk Governorate. The samples were collected from individuals aged 2-45 years between October 27, 2022, and January 3, 2023. Specialized doctors diagnosed the symptoms and recorded the patients' clinical information.

Isolation and identification of bacteria

The samples were placed on blood agar, Mannitol salt agar, and MacConkey agar and then kept in an incubator at a temperature of 37°C for 24 hours. The diagnosis of bacteria occurs by several steps such as growth on selective media like (eosin methylene blue agar), subsequently, microscopic examination after gram stain application, then biochemical tests like(IMViC, Oxidase, Catalase, coagulase, motility, and the ability to create H2S and gas on Kligler iron agar), and examination the ability of bacteria to produce enzymes like(β - Lactamase, urease, protease, and hemolysin enzymes), finally confirmed the diagnosis by VITEK 2 Compact System (Biomerieux, France).

Morphological detection of virulence factors

2.3.1: β - Lactamase production test

The iodometric spot method was employed by placing a square piece of dry filter paper in sterilized Petri dishes. Next, 0.02 g of Penicillin G powder was carefully moved to the middle of the paper, followed by the addition of a few drops of distilled water. A portion of the mixture was subsequently transferred. The dishes containing the active young colony were combined with penicillin in the center of the leaf. They were then left at room temperature for 10 minutes. After that, 3-5 drops of Gram Iodine solution were applied to the entire leaf. The result was observed 5 minutes after adding iodine. A positive result was indicated by the presence of a white spot in the center of the leaf.

2.3.2: Hemolysin production test

The isolates under study, to be tested for their ability to produce hemolytic enzyme, were cultured by stripping method on blood agar media and incubated at 37 °C for 24 hours. The positive result was in the form of decomposition and the appearance of a transparent area in the case of complete decomposition or the form of a green belt in the case of partial decomposition around the colonies [9].

2.3.3: Biofilm Production Test

Tube Method

The bacteria were cultured in test tubes containing liquid BHI medium and 1% glucose and then incubated at a temperature of 37 °C. Following 24 hours, the tubes were drained and subsequently rinsed meticulously with distilled water. They were then allowed to air dry before being treated with a 1% crystal violet solution for 30 minutes. The surplus dye was subsequently eliminated and the tubes were rinsed again. Upon drying the tubes, the presence of biofilm formation was detected. A positive result was determined when thin films were found on the walls and bottom of the tubes [10].

2.3.4: Protease production test.

Skimmed milk medium was used to detect the ability of the isolates under study to produce protease enzyme, where a suspension of bacteria was prepared by taking a single colony and placing it in 5 ml of nutrient broth medium and incubating at 37 °C for 18-24 hours, 20 microliters were transferred from each It stuck to holes with a diameter of 6 mm, which was pierced by a cork borer on the surface of the agar, and the dishes were placed in the incubator at 37 °C for 24-48 hours, then the result was observed, the appearance of a transparent degradation zone around the pits is evidence of the bacteria's ability to degrade proteins.

2.3.5: Urease production test

The tubes containing urea agar were inoculated with the bacterial isolates under study and incubated at a temperature of 37 °C for 24 hours. The change in the color of the medium from yellow to pink is evidence of the positiveness of the test. To raise the PH value of the medium.

2. Molecular Detection of Virulence Factors for Isolated Bacterial Species.

Genes responsible for encoding the production of virulence factors were molecularly detected including β-hemolysin of bacteria *Staph aureus*, biofilm production of *E. coli* bacteria, and protease enzyme production of *Kocuria kristinae* bacteria were also investigated Urease enzyme production of *P. mirabilis* bacteria.

3.1.Extraction of plasmid DNA from bacteria

Geneaid Presto Mini gDNA Bacteria Extraction Kit was used.

The reaction was done separately for each component. The mixture included 10µl GoTaq® Green Master Mix (Promega, USA), 3µl DNA template, 1µl forward and reverse primers, and 5µl nuclease-free water. Tables 1 and 2 show primer sequences, PCR product sizes, and reaction parameters. The PCR findings were electrophoresed on a 2% agarose gel.

Table 1: Oligonucleotide Primers Used for Virulence Genes Amplification.

Primer	Nucleotide Sequence	Product (bp)	Bacteria	Description	Reference
hlB	**F:GTG CAC TTA CTG ACA ATA GTG C** **R: GTT GAT GAG TAG CTA CCT TCA GT**	**309**	***Staph. aureus***	**Hemolysin**	**Aljelehawy *et al.*, 2021 [10]**
ndvB	**F:GGA CAG GGC AAG GTT TAT T** **R:GGT TAT ACT CAG CAG CAC TAT C**	**950**	***E. coli***	**Biofilm**	**Zaiean & Maaroof ,2020 [11]**
ureR	**5'TGAGTGCGAAATTGCGATGG3'** **5'GCGGTTTATCACGAAGGGGT3'**	**359**	***P. mirabilis***	**Urease**	**AL-Atrash and Yasseen,2017 [12]**
Prot_clp	**F: TTCGAGGACCGCATCAT** **R: CTGCGGCTTGTTGATCTT**	**602**	***K. kristinae***	**Protease**	**Al-Douri and Maarouf,2018 [13]**

Table 2: PCR Conditions For Each Primer.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extention
hlb	**94°/5min**	**95°/30sec**	**52°/45sec**	**72°/60sec**	**72°/6min**
	1cycle	**35cycle**			**1cycle**
ndvB	**95°/3min**	**95°/45sec**	**57°/60sec**	**72°/60sec**	**72°/7min**
	1cycle	**35cycle**			**1cycle**
ureR	**94°/2min**	**94°/60sec**	**58°/60sec**	**72°/60sec**	**72°/5min**
	1cycle	**35cycle**			**1cycle**
Prot_clp	**95°/5min**	**95°/45sec**	**58°/45sec**	**72°/45sec**	**72°/5min**
	1cycle	**35cycle**			**1cycle**

3. RESULTS

3.1. Isolation :

The results showed that the number of samples in which bacterial growth appeared on the media used MacConkey agar, Mannitol salt agar, and Blood agar reached 91 samples.

3.2. Detection of virulence factors:

The ability of some isolated bacteria to produce some virulence factors that increase their susceptibility to causing various infections in humans was investigated for the molecular detection of the genes responsible for the production of these factors and to confirm the molecular diagnosis. The bacterial species included *E. coli*, *Morganella morganii*, *P. mirabilis*, *Klebsiella pneumoniae*, and *staph. aureus*, *Kocuria kristinae* and *Staph. lentus*, these factors included their ability to analyze blood on blood agar media and the production of urease enzyme, and the presence of a biofilm was investigated in the dependent isolates, and the examination was carried out using the in vitro method, and in vitro, method gave higher accuracy, so its results were approved.

The results showed the ability of two *Staph. aureus* bacteria isolates by 100% on biofilm formation, as in Figure (1. b). and one isolate was 50% urease-producing (Figure 2. a). Also, the two isolates were 100% soluble in blood, β-type hemolytic, as in Figure (4. a).

As for two isolates of *Staph. lentus* did not show their ability to produce biofilm Figure (1. b), While no isolates of this bacteria showed the ability to produce urease (Figure 2. c).

As for the *Kocuria kristinae* bacterium, the results showed that one isolate, 100% formed a biofilm, and one isolate 100% was a beta-type hemolytic. While one isolate showed 100% of these bacteria the ability to produce protease enzyme (Figure 5. b), One isolate was 100% beta-lactamase-producing(Figure 3. b), and one isolate was 100% urease-producing (Figure 2. b). All isolates showed their ability to form a biofilm by 100%. While 4 isolates showed 80% of their ability to produce a

gamma-type lysate, and one isolate showed 20% of a β-beta lysate.

As for *Proteus mirabilis***,** 3 isolates showed 60% ability to produce the beta-lactamase enzyme (Figure 3. a). Also, 4 isolates showed 80% of this bacteria the ability to produce the enzyme protease (Figure 5. e).

As for *Morganella morganii*, one isolate showed 20% of its ability to form a biofilm, while 3 isolates had 60% γ-hemolytic and two 40% β-hemolytic (Figure 4. b). While two isolates showed a 40% capacity for these bacteria to Protease enzyme production.

The results showed the ability of 3 *E.coli* isolates, a percentage of 75% to form a biofilm.

Two isolates of *Klebsiella pneumoniae* bacteria showed 50% of their ability to form a biofilm. While 3 isolates had 75% blood lysate type gamma γ and one isolate with 25% blood lysate type beta β, while 3 isolates showed 75% of their ability to produce urease enzyme.

Table 3: The number and percentage of the ability of Gram-positive and Gram-negative bacteria species under study to produce virulence factors

Isolated bacterial species	Number of isolates	Biofilm		Hemolysin						Protease		β-lactamase		Urease	
				α		β		γ							
		No.	%	No	%	No.	%	No.	%	No.	%	No.	%	No.	%
Staph. aureus	2	2	100			2	100			0	0	1	50	1	50
Proteus mirabilis	5	3	60			1	20	4	80	4	80	3	60	3	60
Morganella morganii	5	1	20			2	40	3	60	2	40	5	100	3	60
E.Coli	4	3	75			4	100			1	25	4	100	4	100
Klebsiella pneumonia	4	2	50			1	25	3	75	3	75	4	100	3	75
Kocuria kristinae	1	1	100			1	100			1	100	1	100	1	100
Staph. lentus	2	0	0			2	100			2	100	2	100	0	0

Figure 1. a: The inability of *Staph lentus* bacteria to form a biofilm in the tube method.
b: The ability of *Staph aureus* to form a biofilm in vitro

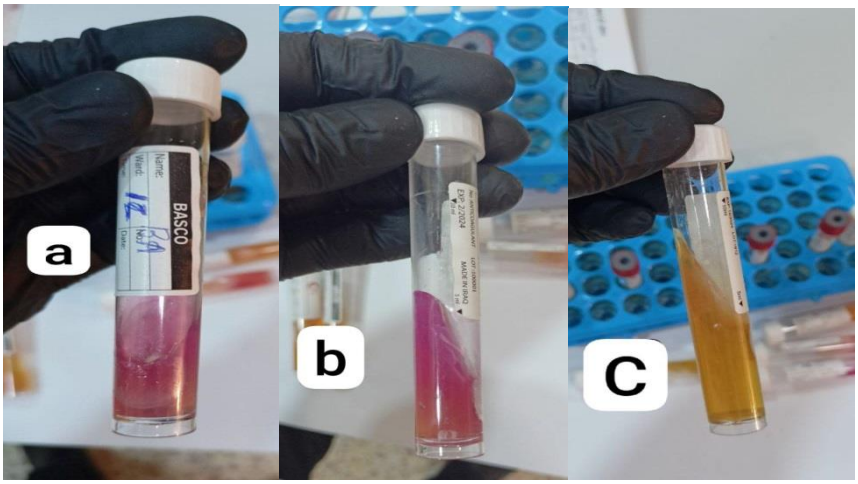

Figure 2.a: The ability of *Staph. aureus* to produce urease enzyme, as the color of the medium changed from yellow to pink.
b: The ability of *K. kristinae* to produce urease enzyme, as the medium changed color from yellow to pink.
c: The inability of *Staph. lentus* to produce the urease enzyme if it did not change the color of the medium.

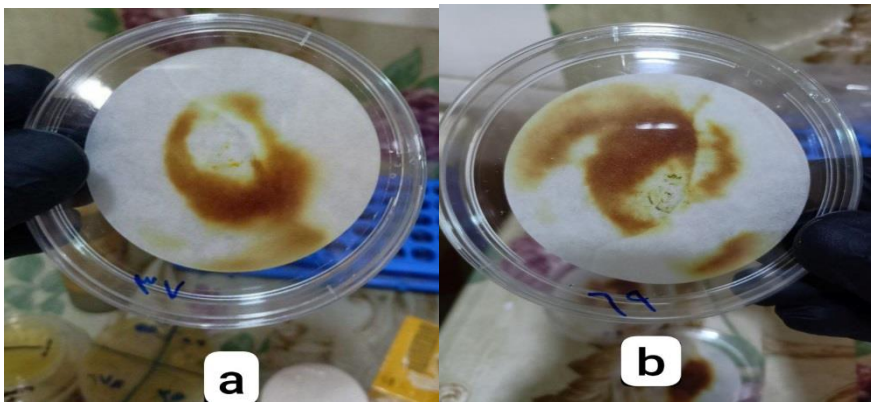

Figure 3.a: The ability of *Proteus mirabilis* to produce beta-lactamase enzyme by iodide stain method.
b: The ability of *Kocuria kristinae* to produce beta-lactamase enzyme by iodide stain method.

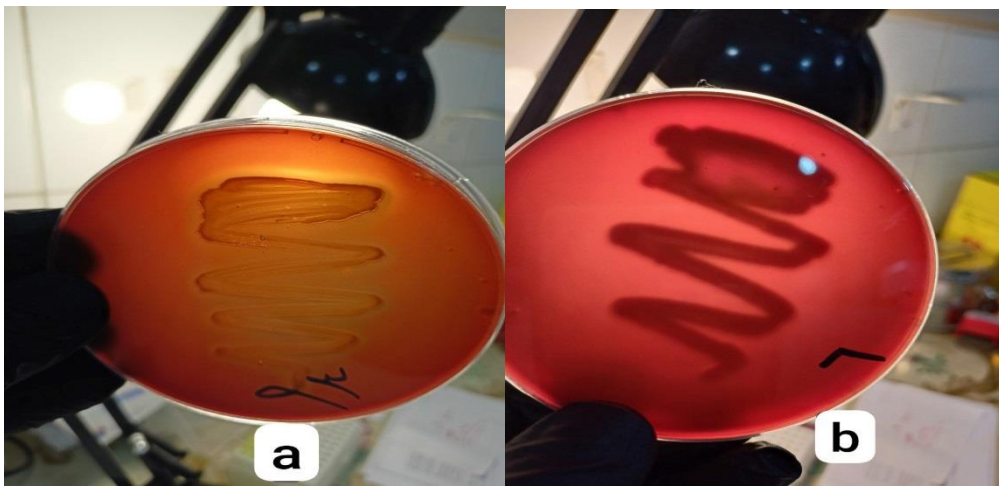

Figure 4. a: The transparent areas of β-hemolysin degradation of *Staph aureus* bacteria.
b: hemolysis of γ-haemolysin of *Morganella morganii* bacteria.

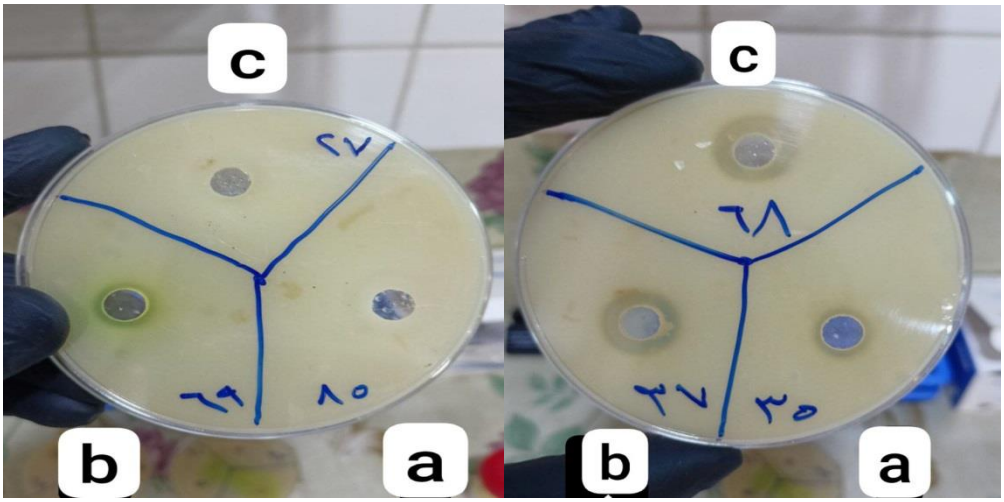

Figure 5: Zones of transparent decomposition around the pits which is indicative of protease production by b: *K. kristinae*, d: *K. pneumoni*, e: *P. mirabilis*, f: *Staph. Lentus*
The negative result is the inability to produce protease by a: *Staph aureus*. c: *E.coli*.

Plasmid DNA extraction

Plasmid DNA was extracted from seven isolates, two for each *Staph. aureus*, *E. coli*, *P. mirabilis* bacteria, and one isolate of *K. kristinae* bacteria. In the extraction procedures, a group of chemicals used in the extraction solutions was used, as each of these materials eliminates one of the unwanted components of the bacterial cell while at the same time preserving the DNA being the target of the extraction process. The success of the nucleic acid extraction process was verified by electrophoresis of nucleic acid on a 1% agarose gel. The results of electrophoresis on the agarose gel showed that all samples contained DNA as shown in (Figure 6). The concentration and purity of DNA samples extracted from the isolates under study were determined using a Nanodrop spectrophotometer as shown in (Table 4). The nucleic acid concentrations were adjusted to a concentration not exceeding (50 ng/µl) using deionized distilled water free of nucleotides. The nucleic acid was used after standardization in the polymerase chain reaction (PCR).

3.3. Virulence Genes Detections

The polymerase chain reaction (PCR) was used to detect the presence of some genes encoding virulence factors (*hlaB*) in *Staph aureus* bacteria,

(*ndvB*) in *E. coli*, (*UreR*) in *P. mirabilis*, (*Prot_clp*) in *K. kristinae*, then electrophoresis was carried out, and DNA bundles appeared The hlaB gene was amplified using a hlaB-specific primer. The agarose gel analysis of the PCR products showed that the hlaB gene, which codes for hemolysin production, was present in both of the Staph. aureus bacteria samples, which means that it happened in both of them. This gene showed up on an agarose gel in a bundle that was (309bp) in size, as in Figure (7).

The gene Prot_clp was amplified using the specific primer Prot_clp. The electrophoresis results on an agarose gel of the PCR products indicated the existence of the Prot_clp gene, which codes for the production of a protease, in one isolate of K. kristinae. The incidence rate of this gene was found to be 100%. The gene was observed as a cluster on the agarose gel, measuring 602 base pairs in size, as in Figure (8).

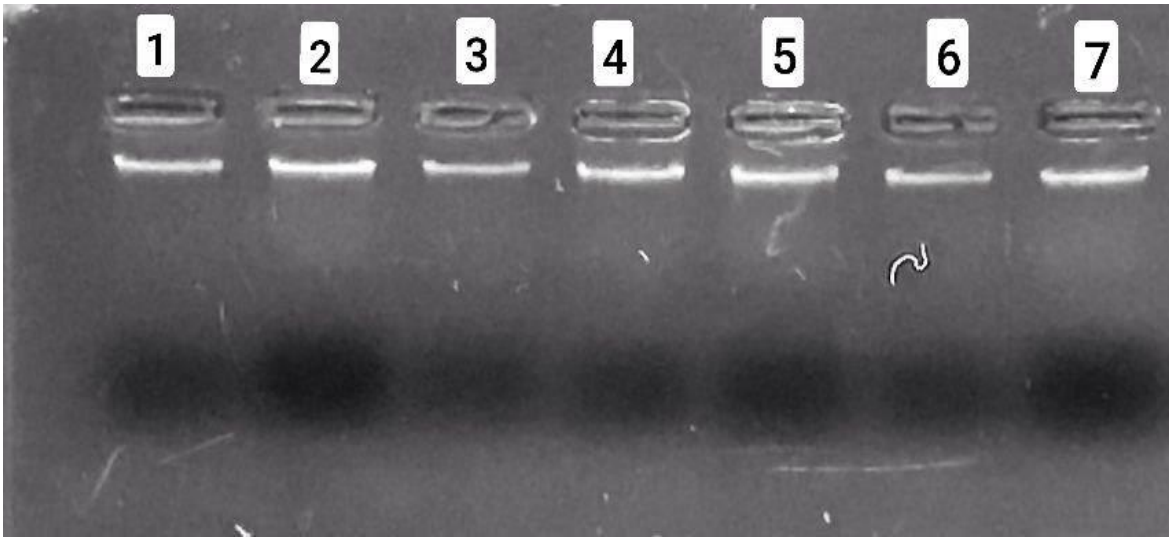

Figure 6: DNA transfer of the isolates under study on an agarose gel at a concentration of 1%. *P. mirabilis*(1-2), *E. coli*(3-4), *K. kristinae*(5), *Staph. aureus*(6-7)

Table 4: Concentration and purity of isolates after DNA extraction

Isolates	Concentration	Purity
Staph. aureus	**44,3**	**1,8**
Staph. aureus	**39,6**	**1,69**
K. kristinae	**24,7**	**2,2**
E. coli	**48,46**	**1,81**
E. coli	**56,1**	**1,76**
P. mirabilis	**26,1**	**1,43**
P. mirabilis	**26,21**	**1,61**

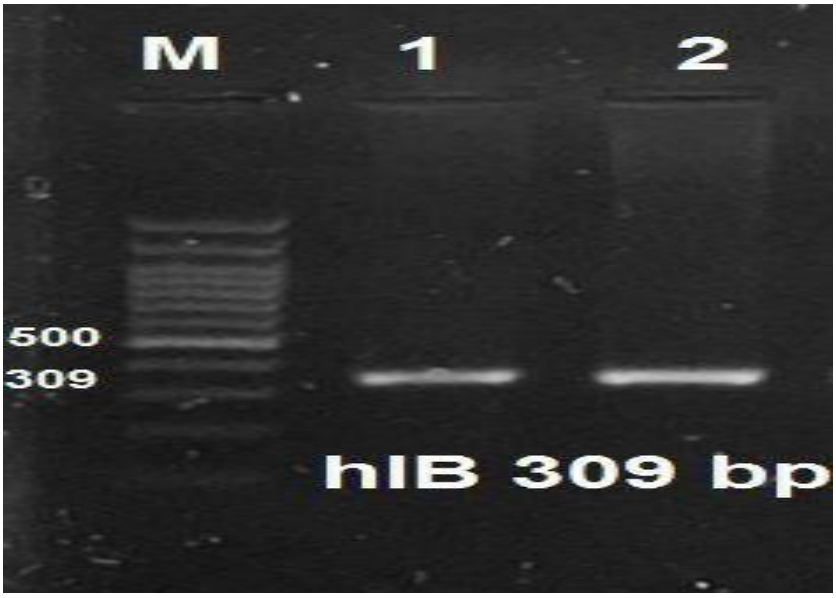

Figure 7: Electrophoresis on an agarose gel of the products of the polymerase chain reaction (PCR) of the DNA of *Staph. aureus* using the specialized primer for the *hlaB* gene, track 1 at 309 bp in size representing the *hlaB* gene
M: DNA ladder (100bp)

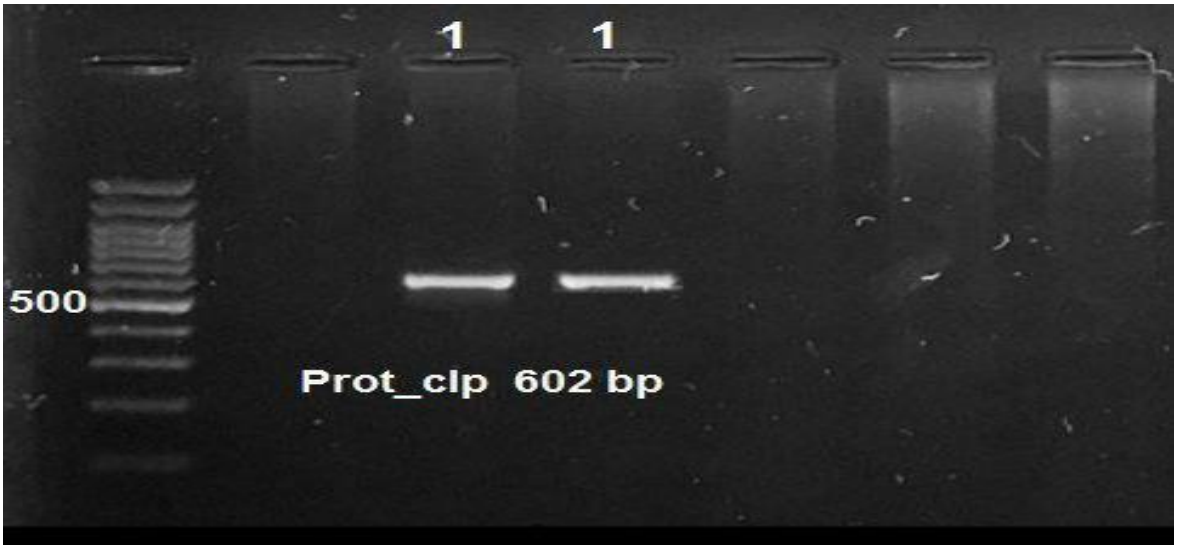

Figure 8: Electrophoresis on an agarose gel of the products of the polymerase chain reaction (PCR) of the DNA of *K. kristinae* using the specialized primer for the *Prot_clp* gene, track 1 at 602 bp in size representing the *Prot_clp* gene.
M: DNA ladder (100bp)

The ndvB gene was amplified using the specialized initiator ndvB. Electrophoresis on an agarose gel of the PCR products showed that the ndvB gene, which codes for biofilm formation, was present in only one sample of E. coli, which is a 50% chance. On the agarose gel, this gene showed up as a group. The size (950 bp), as shown in Figure (9), the loss of the gene responsible for encoding biofilm formation in the second isolate of *E.coli* bacteria can be attributed to a genetic mutation in the sequences of nitrogenous bases that led to the incomplete translation process, or it can be attributed. This indicated that the optimal conditions for the PCR reaction did not match the replication and elongation stages in terms of temperature or time.

The specialized initiator UreR was used to amplify the UreR gene. The PCR products were electrophoresed on an agarose gel, and the results showed that the UreR gene, which codes for the formation of urease, was present in two isolates of P. mirabilis with a 100% incidence rate. This gene appeared as a bundle with a size of 359 bp on the agarose gel, as in Figure (10).

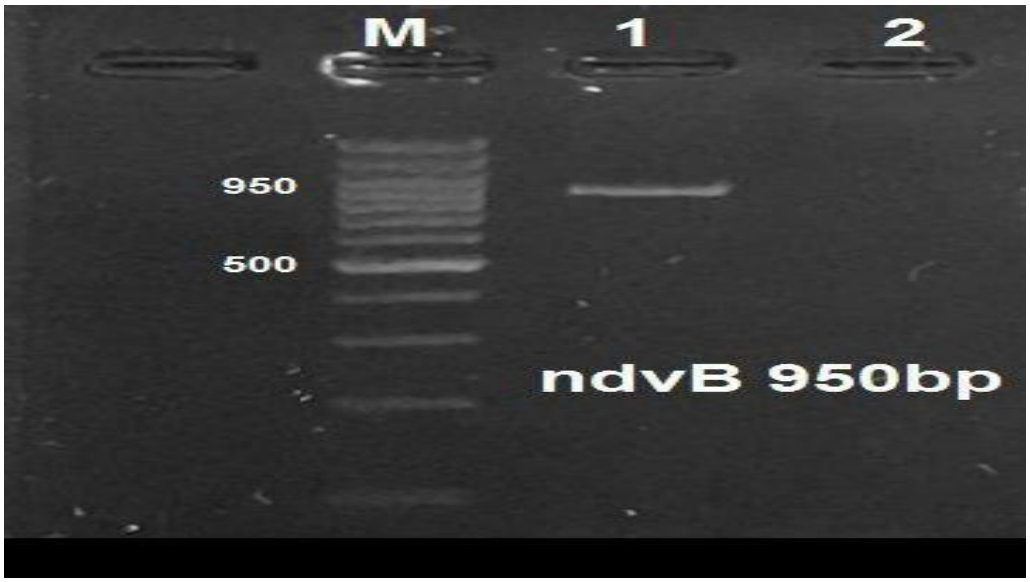

Figure 9: An agarose gel electrophoresis was performed on the polymerase chain reaction (PCR) products of E. coli DNA using a specific primer for the ndvB gene in pathway 1. The PCR result had a length of 950 base pairs, representing the ndvB gene. The DNA ladder used as a reference had a length of 100 base pairs.

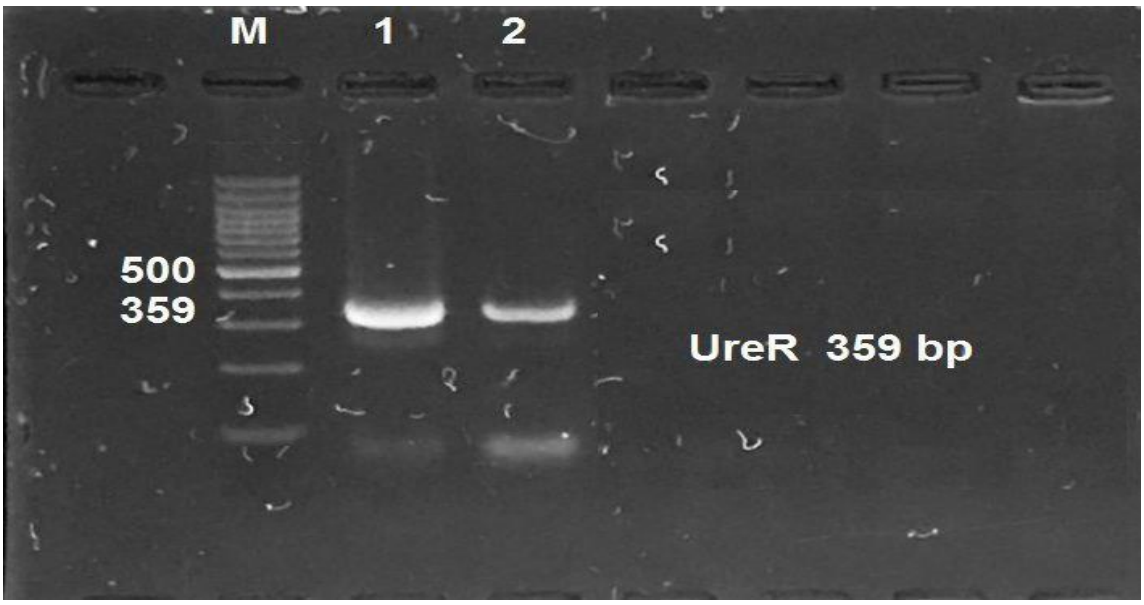

Figure 10: PCR results of P. mirabilis DNA are subjected to electrophoresis on an agarose gel. The UreR gene is represented by tracks 1-2 at 359 kb using the particular primer for this gene. M: 100 base pair DNA ladder

5. DISCUSSION

The percentages with a rate of 91%, were close to the percentage of several previous studies [15- 19] which were 98.2% - 93.2% - 92.8% - 93% - 95.5% respectively, while 9 samples did not give any growth of 9% of the total samples, The lack of bacterial growth can be attributed to the presence of a non-bacterial pathogen, such as a virus, fungus, anaerobic bacteria, or mycoplasmas that cannot be detected using the standard aerobic bacterial culture methods employed in this study. These pathogens may require specific culture media and special conditions for isolation. Alternatively, the absence of bacterial growth may be due to the administration of antibiotic doses by the patients, which was documented in the patient records[20]. The in vitro method gave higher accuracy, so its results were approved. which agreed with [21,22] In addition to its ability to produce protease and beta-lactamase enzymes.

This result differed from the result of [14], as the percentage of isolates forming biofilm was lower by 78.1%, The results of the studied isolates coincided with the results of the study of [23], as the isolates showed their ability to analyze blood type β-hemolysin by 100%. This result agreed with the result of the researchers [24], as they mentioned that no isolates produced *Staph. lentus* urease enzyme. This result differed from the results of the study by the researcher [25], as they showed that it is not a blood analyzer. The results showed the ability of 3 isolates of the bacteria *Proteus mirabilis* with a rate of 60% to form a biofilm. Our results differed from the results of the study of [26]. This result did not agree with the result of [27], where *Proteus mirabilis* isolates showed 97.3% of their ability to produce a lysate. blood beta and 2.7% of α-hemolysin. The results of our study differed from the study of [28], noting that Proteus species produce 100% beta-lactams.

The findings of the present investigation align with the findings of the previous study conducted by [14], as we demonstrated that all strains of Proteus mirabilis bacteria exhibit a positive reaction in the Protease test. This result closely aligns with the findings of the study conducted by [29], where it was

demonstrated that the isolates exhibited a biofilm production rate of 61.53%. Four isolates exhibited complete beta-type hemolysis. The findings of our investigation aligned with the research conducted by the researcher [30], which demonstrated that *K. pneumoniae* bacteria exhibited a 50% capacity to develop a biofilm. Conversely, our study yielded contrasting results compared to the findings of the researcher [31,32].

This result converged with the study of [14], as they found this initiator in 9 out of 10 isolates with a rate of 90%, and they converged. This result is also consistent with the study of [30], as they found this initiator in 32 out of 33 isolates, with a rate of 90%. and these results coincided with the study of [13,14], as they found that all isolates produced the *UreR* gene.

6. CONCLUSION

The majority of the bacteria species under study showed their ability to produce virulence factors with a high percentage of the enzyme hemolysin and.β-lactamase, Molecular detection of the genes encoding the production of virulence factors is under study, which included *hlaB* in *Staph. aureus ndvB* in *E. coli*, *UreR* in *P. mirabilis*, and *Prot_clp* in *K. kristinae*, its presence was shown at a rate of 100%, except for the *ndvB* gene, which was present at a rate of 50%

Conflict of Interest

The authors declare that they have no conflict of interest.

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