



Isolation and Molecular Diagnosis of Bacteria Causing Bacteremia, and Study Their Relationship with Kidney Functions

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ABSTRACT

Bacteremia is a life-threatening and a critical infection. In this study, 250 blood samples were collected from patients and directly cultured in brain-heart infusion broth and incubated aerobically, which was considered the primary culture. After observing any changes in the medium in the following day, a secondary culture was performed on basal blood agar and MacConkey agar. The colonies and cells morphologies were examined. Molecular diagnosis was then carried out based on the 16S rRNA gene, Following by nucleotide sequencing. 19 Gram-negative and 16 Gram-positive bacterial strains were identified. The isolated bacteria were as follows: *Klebsiella pneumoniae* (6), *Shigella sonnei* (5), *Serratia surfactantfacie* (2), *Pseudomonas aeruginosa* (2), *Burkholderia paludis* (1), *Escherichia coli* (2), *Enterobacter spp.* (1), *Staphylococcus aureus* (2), *Staphylococcus haemolyticus* (2), *Staphylococcus epidermidis* (11), and *Micrococcus yunnanensis* (1).

Bacterial subspecies of *Klebsiella pneumoniae* was identified through the use of the Voges-Proskauer test and malonate consumption test. It was found that the dominant subspecies was *K. pneumoniae subsp. ozaenae*, with 5 strains out of 6 showing negative results for both tests. Only one strain belonged to *K. pneumoniae subsp. pneumoniae*, which was positive for both tests. The correlation between bacteremia and kidney function was investigated, as well as the effect of gender. The levels of urea and creatinine were measured in patients diagnosed with bacteremia and compared to healthy controls. Statistical analysis revealed neither significant differences in kidney function between patients and healthy individuals, nor significant differences between male and female patients or between healthy controls.

Keywords: *Klebsiella subspecies*, malonate consumption test, urea, creatinine

INTRODUCTION

Bacteremia is a global health concern and represents a serious infection characterized by high morbidity and mortality rates, particularly among children and the elderly. This depends on several factors, including the virulence of the pathogen and host-related factors (Duan *et al.*, 2021).

Healthy individuals' blood must not contain any bacteria; therefore, isolating bacteria from blood samples is typically an indication of a serious invasive infection that requires immediate treatment with antibiotics. Bacteria in the blood can significantly affect the patient's life. Most cases are caused by a variety of pathogenic agents, including *Staphylococcus* spp., *Streptococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas* spp. (Diekema *et al.*, 2019). The infection may be localized to a specific site in the body, while the movement of bacteria facilitates their entry into the bloodstream (Garnica *et al.*, 2021).

Among the bacterial genera responsible for bacteremia, *Klebsiella pneumoniae* is a Gram-negative, rod-shaped bacterium that belongs to the Enterobacteriaceae family. It is lactose-fermenting, which results pink colonies on MacConkey agar. Their colonies are characterized by a mucous appearance due to the secretion of a capsule around the bacterial cells.

Renal dysfunction may be observed during acute bacteremia, where kidney failure is defined as a rapid decline in kidney function, indicated by increased serum creatinine levels or reduced urine output (Kellum *et al.*, 2008).

The aim of this research is to isolate the bacteria responsible for bacteremia and to diagnose them molecularly using the 16S rRNA gene, followed by an assessment of their impact on renal function through the measurement of urea and creatinine levels.

MATERIALS AND METHODS

Sample Collection

A total of 250 blood samples were collected from patients in the intensive care unit, Bacteriology department, and wards of hospitals in Mosul city from (Mosul General Hospital, Ibn Sina Teaching Hospital, Al-Jamhuri Hospital, and Al-Salam Hospital) during the period from August to October 2024, Based on the approval of the scientific and ethical research committee at its session numbered (260) held on 2/ 10/ 2024 on the research project numbered (2024163).

5 ml of blood were collected from each patient. 1 ml of blood was mixed with 5 ml of Brain Heart Infusion (BHI) broth in a special tube for blood culture. The tubes were then incubated aerobically at 37°C for 7 consecutive days as an initial culture. During this period, any changes, such as turbidity or hemolysis in the broth, were monitored. If such changes occurred, a subculture was performed, subsequently, a subculture was performed by taking 0.1 ml from the initial culture after gently mixing the sample by turning the tube up and down several times until it became homogeneous. This sample was then cultured on blood agar and MacConkey agar and incubated for 24 hours at 37°C under aerobic conditions. Then, observation of the presence or absence of growth.

The remaining 4 ml of blood was placed in gel tube and centrifuged to separate the serum, which was then stored at -18°C until use.

Bacterial Identification:

Phenotypic Identification:

After inoculating on blood agar and MacConkey agar, the growing colonies were observed based on their color, shape, size, edges, texture, height, and consistency (de la Maza *et al.*, 2020). Gram stain was performed to examine the bacterial cells to determine whether they spherical or rod-shaped and to identify their staining characteristics, either red or purple, depending on their reaction to the stain.

Molecular Identification:

The unknown isolates were cultured on LB broth; molecular identification was carried out based on the 16S rRNA gene. The first step in the molecular diagnosis was DNA extraction from the bacterial isolates (Geneaid kit /Taiwan), depending on the company's manufacturing instructions.

The purity and concentration of the extracted DNA were measured using a Nanodrop device. The absorbance was read at a wavelength of 260/280 nm (Desjardins *et al.*, 2011).

PCR Protocol for Diagnosis:

16S rRNA gene primers were used, which were obtained from Macrogen Company (South Korea) according to (Dos Santos *et al.*, 2019). (Table 1) shows the sequences of the forward and reverse primers for the 16S rRNA gene and their molecular size.

Table 1: the sequences of the forward and reverse primers for the 16S rRNA gene and their molecular size.

Gene name	Primer	Sequence 5'-3'	Size(bp)
16s rRNA	F27	AGAGTTTGATCCTGGCTCAG	1500
	R1492	AGAGTTTGATCCTGGCTCAG	

PCR Reaction Mixture Preparation:

A PCR reaction mixture of 30 μ L was prepared, consisting of the extracted DNA, primers, Master Mix (2x), and nuclease-free water as shown in (Table 2).

Table 2: PCR Reaction Mixture with final volumes and concentrations

Product	Volume in microliter	Final Concentration
Master Mix (2)	15	1x
Forward Primer	1.5	1 micromole
Reverse Primer	1.5	1 micromole
DNA Extracted	1-4	<250 ng
Nuclease free water	Volume completed to 30 microliters	
Total Volume	30	

The reactions were performed on GeneAmp PCR System 9700 (Applied Biosystems™) thermocycler with the program: 4 min at 96 °C, followed by 30 cycles of (30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C), and a final extension step at 72 °C for 10 min.

Electrophoresis (2%) agarose gel was prepared, and 3 μ L of red safe dye from Add Bio (South Korea) was added. A DNA ladder and PCR product were also added to the gel after hardening.

DNA Sequencing:

The PCR products were sent to Macrogen (South Korea) for DNA sequencing using the Sanger method with the forward primer, the results of sequencing were compared on the NCBI website through BLAST to determine the identity of our isolates by matching them with global strains.

Detection of subspecies of *K. pneumoniae* Strains:

To determine the subspecies of *K. pneumoniae* strains, two tests were conducted: The Voges-Proskauer (VP) test, based on (Collee *et al.*, 2008), and the malonate utilization test, based on (de lamaza *et al.*, 2022).

Voges-Proskauer Test (VP): The peptone water medium was prepared. The pH was adjusted to 6.9, and the broth was distributed into tubes, sterilized by autoclaving. Once the medium cooled, it was inoculated with *K. pneumoniae* strains and incubated for 48 hours at 35°C. Reagents, were added as follows: first, 9 drops of alpha-naphthol reagent were added and mixed. Then 3 drops of potassium hydroxide reagent were added, mixed again, and the tube was gently shaken for 30 seconds, exposing it to oxygen to facilitate the color reaction. The results were read after 15 minutes after the addition of the reagents.

Malonate Utilization Test: The medium (malonate broth) was prepared with the components, and the pH was adjusted to 6.7. The broth was distributed into tubes, sterilized by autoclaving, and inoculated with *K. pneumoniae* strains. The tubes were incubated for 24 hours at 37°C. After incubation, the results were observed by noting the color change from blue to dark blue, which is referred to as Prussian blue.

Renal Function Tests

Serums were collected from the samples that showed bacterial growth on the plates. Kidney function tests were conducted, specifically for urea and creatinine to assess kidney function depending on the kit (FUJIFILM Corporation- Japan). The tests were performed using the Fujifilm device. After thawing the serum and placing it in a small tube, it was inserted into the designated slot in the Fujifilm device. The test discs for each assay were placed in the appropriate slot in the device. After recording the sample information and pressing the start, the barcode for each test was scanned by the device, and the results were displayed within a few minutes.

Statistical analysis: The data were analyzed using a simple experimental design with a completely randomized design, and a t-test was applied to compare the treatments between the data for both patients and healthy individuals, for both genders.

RESULTS AND DISCUSSION

After collecting 250 blood samples from patients suspected of having bacteremia from hospitals in Mosul city, it was found that 216 samples did not show any growth, accounting for approximately 86%. In contrast, growth was observed in 37 samples, which was about 16%. The samples which showing the growth of a single bacterial species were included, while samples showing the growth of more than one bacterial species were excluded and that occurred with two samples. The diagnosis was done using PCR and through sequencing alignment with NCBI, a total of 35 bacterial isolates were obtained, of which 19 isolates were Gram-negative with 7.6%, and 16 were Gram-positive, equal to 6.4%.

The presence of the 16s rRNA gene bands was observed in all isolates, as shown in Fig. (1).

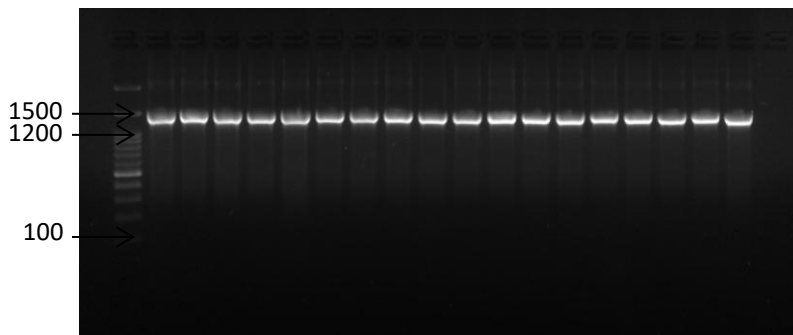


Fig. 1: the electrophoretic of the polymerase chain reaction product of the 16s rRNA gene for bacterial isolates on agarose gel, with an applied voltage of 100 volts for one hour.

The most common bacteria identified among the Gram-negative isolates were *Klebsiella pneumoniae*, which led with six isolates, followed by *Shigella* with five isolates, 2 isolates of *Serratia surfactantfaciens* were isolated with different species, and one isolate of *Enterobacter*, 2 isolates of *Burkholderia spp*, and 2 isolates of *Escherichia coli*, one isolate of *Pseudomonas aeruginosa*.

Among the Gram-positive bacteria, there were 11 isolates of *Staphylococcus epidermidis*, 2 of *Staphylococcus haemolyticus*, 2 of *Staphylococcus aureus*, and one isolate of *Micrococcus spp*.

The sequencing of the 16S rRNA gene was performed. The molecular diagnostic results for the bacterial isolates showed sequence identity percentages ranging from 97% to 100%.

The highest percentage of bacteremia was observed with *Klebsiella pneumoniae*, accounting for 31.5%, followed by *Shigella* at 26.3%, *Serratia* at 10.5%, *Pseudomonas* at 10.5%, *Burkholderia*

at 5.2%, *Staphylococcus haemolyticus* at 12.5%, *Staphylococcus aureus* at 12.5%, *Staphylococcus epidermidis* at 68.7%, and *Micrococcus* at 6.2%.

These percentages differed from the findings of (Al-Wandawy *et al.*,2023), where *Klebsiella pneumoniae* accounted for 9.52%, *Pseudomonas* for 52.38%, *Staphylococcus haemolyticus* for 9.1%, and *Staphylococcus epidermidis* for 7.1%. Notably, *Staphylococcus epidermidis* was found to be the most prevalent Gram-positive bacteria, which is often associated with skin and is not typically pathogenic; its presence may be due to improper skin disinfection during blood collection.

Our results disagree with (Matos *et al.*,2023), who reported that the percentage of Gram-positive bacteria isolation was around 21%, whereas in our study, the percentage of Gram-positive bacteria was approximately 6.4%. However, our findings agreed with (Padmajal, *et al.*,2022), where the percentage of Gram-negative bacteria was higher than that of Gram-positive bacteria. Specifically, the percentage of Gram-negative bacteria was 52.7%, and Gram-positive bacteria were 47.3%, in addition in their study, *K. pneumoniae* accounted for 33.3%, *P. aeruginosa* for about 12.28%, while *S. aureus* represented about 35.0%. *K. pneumoniae* represented about 31.5%, *P. aeruginosa* accounted for 10.5%, and *S. aureus* was 12.5%.

In immunocompromised individuals, infections can spread into the bloodstream, leading to bacteremia or septicemia, often following pneumonia. The gastrointestinal system is also a major source of these infections (Ku *et al.*, 2017). *Escherichia coli*, a Gram-negative bacterium belonging to the Enterobacteriaceae family, normally resides in the human intestines without causing harm. However, it can lead to intestinal infections that may result in serious invasive infections such as bacteremia (Skogberg *et al.*, 2008).

Burkholderia is an aerobic, Gram-negative bacterium that can persist for long periods in water and is naturally resistant to many antimicrobial agents (Bharara *et al.*, 2020). It is an opportunistic pathogen often acquired in healthcare settings and can cause severe infections in children. *Serratia surfactantfaciens* is a Gram-negative bacterium that has occasionally been responsible for healthcare-associated infections, including bacteremia and endocarditis, particularly in individuals who inject drugs (Phadke and Jacob, 2016). *Staphylococcus aureus* is a Gram-positive, facultative anaerobic coccus known for its unique pathogenic characteristics, such as its ability to migrate into the bloodstream and produce adhesive substances that help it attach to both living and non-living surfaces, forming biofilms (Jenkins *et al.*, 2015).

Staphylococcus epidermidis is the most studied member of the *Staphylococcus species*. It can be isolated from all skin microenvironments, including dry, moist, and oily areas, as well as from the feet. However, there is significant strain diversity among these isolates, particularly concerning virulence factors related to the host. while two isolates of bacteremia in the study (Al Jarjary and Alsammak, 2023).

Identification of the *K. pneumoniae* Subspecies:

After inoculating the peptone water medium with six *K. pneumoniae* strains and incubating them, the addition of reagents resulted color changed in only one broth to pink, which corresponded to one strain. The remaining strains showed no color change, thus testing negative for this assay, as illustrated in Fig. (2).



Fig. 2: The color changed to pink due to the fermentation of glucose

The VP detects the organism's ability to convert the acid products to acetoin and 2,3-butanediol compound. Organisms capable of using the VP pathway produce a smaller amount of acid during glucose fermentation and therefore do not produce a color change when the Methyl Red indicator is added. A secondary reagent is added, alpha-naphthol, followed by potassium hydroxide (KOH); a positive test result indicated by a pink color complex (Tille, 2022).

For the malonate test, the same strain that tested positive in the Voges-Proskauer test, was also positive for this test, with the medium turning dark blue (Prussian blue), as shown in Fig. (3). The five other strains, which did not show any color change in malonate taste were negative for VP test also.



Fig. 3: The color change of the malonate solution to dark blue (Prussian blue)

The principle of the malonate test: In this test, glucose is consumed, and acids are produced, leading to a decrease in pH and a color change of the indicator. Bacteria capable of utilizing malonate show a positive result and belong to the subspecies *K. pneumoniae* subsp. *pneumoniae*, while bacteria unable to utilize malonate show a negative result and belong to *K. pneumoniae* subsp. *ozaenae*,

Based on the results of both tests, we conclude that one strain only belongs to the subspecies *K. pneumoniae* subsp. *pneumoniae*. The remaining five strains belong to *K. pneumoniae* subsp. *ozaenae*, as these strains tested negative for both the Voges-Proskauer and malonate tests.

In a study by the researchers (Hansen *et al.*,2004), 28 isolates were attributed to the species *K. pneumoniae* subsp. *pneumoniae* and 27 isolates to *K. pneumoniae* subsp. *ozaenae*.

The impact of bacteremia on renal function:

Regarding the renal tests for the samples that showed bacterial growth; after conducting data analysis for both the serum samples of patients (who showed bacteremia) with a total of 23 samples and the healthy controls (with 23 samples), (Table 2) indicates no significant differences between the infected patients and the healthy individuals in terms of urea and creatinine levels, which are markers for kidney function. This suggests that kidney function was not affected by this infection.

Our results in the (Table 2) were different from what Daly reported, Patients with chronic kidney disease (CKD) are more likely to develop cardiac vascular disease (CVD) than their age- matched counterparts in the general population, so that the risk of death from (CVD) is much higher than the risk of eventually requiring dialysis (Daly *et al.*,2007).

Table 2: The Effect of Bloodstream Infection on Urea and Creatinine Levels BUN (Blood Urea Nitrogen) CRE (Creatinine Blood).

Group Statistics ¹							
	parameters	N	Mean	t-value	Sig	Std. Deviation	Std. Error Mean
BUN	patient	23	31.5565	-.378-	.707	+ _20.75381	4.32747
	control	23	33.3478	-.378-	.708	+ _9.22747	1.92406
CRE	patient	23	.8178	-.326-	.746	+ _ .53051	.11062
	control	23	.8565	-.326-	.747	+ _ .20851	.04348

In our study the gender has no impact on the incidence of bacteremia in kidney patients as shown in (Table 3) while the study by Jadaan and Al-Hayali (2023) indicated that there are statistically significant differences among kidney disease patients across both genders.

Table 3: The effect of gender on the number of blood cultures in kidney disease patients BUN (Blood Urea Nitrogen) CRE (Creatinine Blood).

Group Statistics							
	parameters	N	Mean	t-value	Sig	Std. Deviation	Std. Error Mean
BUN	male	11	40.8091	2.223	.037	26.88246	8.10537
	female	12	23.0750	2.134	.056	6.35497	1.83452
CRE	male	11	1.0355	2.009	.058	.71742	.21631
	female	12	.6183	1.921	.083	.06492	.01874

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العزل والتشخيص الجزيئي للبكتيريا المسببة لتجرثم الدم ودراسة تأثيرها على وظائف الكلى

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المخلص

تعد الإصابة بتجرثم الدم من تلك الإصابات المهددة للحياة. في هذه الدراسة جمع 250 عينة دم من المرضى واجري الزرع مباشرة في وسط مرق نقيع القلب والدماغ وحضنت هوائيا وعد ذلك زرعا اوليا، وبعد ملاحظة وجود أي تغيير في الوسط في اليوم التالي تم اجراء الزرع الثاني على وسطي: الدم الاساس وماكونكي اكار. لوحظت اشكال المستعمرات والخلايا تم التشخيص الجزيئي بالاعتماد على الجين 16s rRNA ومن اجراء التتابع النيوكليوتيدي، تم الحصول على (16) سلالة بكتيرية موجبة لصبغة كرام، وعلى (19) سلالة بكتيرية سالبة لصبغة كرام، بحيث كانت البكتيريا المعزولة هي كل من:

Serratia surfactantfaciens (2), *Pseudomonas* ، *Shigella sonnei* (5)، *Klebsiella pneumoni* (6) *Burkholderia paludis* (1), *Escherichia coli* (2), *Enterobacter* (1), *Staphylococcus* ، *aeruginosa* (2) *aureus* (2), *Staphylococcus heamoliticus* (2), *Staphylococcus epidermis* (11), *Microscoccu* .*yunnanensis* (1)

تم تحديد تحت النوع لبكتريا *K. pneumoniae* من خلال اختباري الفوكس بروسكاور واستهلاك المالونيت وتبين ان تحت النوع السائد في دراستنا هو *K. pneumoniae* subsp. *ozaenae*، حيث كان من نصيب 5 سلالات من أصل 6 اذ كانت النتيجة سالبة لكلا الاختبارين، فيما اظهر لدينا في هذا الاختبار سلالة واحدة تعود لتحت النوع *K. pneumoniae* subsp. وكانت موجبة لكلا الاختبارين.

تم دراسة العلاقة بين تجرثم الدم وتأثيره على وظائف الكلى وكتأثير للجنس من جهة أخرى، تم قياس مستوى كل من اليوريا والكرياتينين في المرضى المصابين بتجرثم الدم ومقارنتهم مع اشخاص طبيعيين كسيطرة واجراء التحليل الاحصائي، واطهرت النتائج عدم وجود فروقات معنوية من حيث وظائف الكلى فضلا عن عدم وجود فروق معنوية بين الذكور والاناث المرضى والاصحاء

الكلمات الدالة: *Klebsiella subspecies*، اختبار استهلاك المالونيت، يوريا، كرياتينين.