**Napata College**

**Medical Laboratory Science Program**

**Department of Microbiology**

# Molecular Detection of Aminoglycoside Resistance Gene arm A, ant, Acc3, Acc6 and Aph of *Klebsiella pneumoniae* isolated from clinical specimensin Khartoum State-Sudan 2022.

**arm A, ant, Acc3, Acc6 and Aph** الكشف الجزيئي عن الجينات المقاومة للامينوجليكوسايد للكلبسيله الرئويه المعزوله من العينات السريريه في ولاية الخرطوم-السودان للعام2022

A Thesis submitted to partial fulfillment for the requirement of B.sc degree in Medical Laboratory Sciences (Microbiology)

By:

1. **AbugogoGawKafiWanis.**
2. **AlfatihAlamin Taha.**
3. **AlmojtabaAlfatih Ahmed Ibrahim.**
4. **Suliaman Gorashie Suliaman Khalil.**
5. **Omer Hassan Omer Ahmed.**
6. **Mustafa Yousif Adam Abaker.**

**Supervisor**

**Dr. SahrSaadeldinHagMohamed**

**M.Sc Medical Microbiology**

**Al-Neelain University**

**Co-supervior**:

**Reel GamalAlfadil**

**Bsc, Medical Microbiology**

**Al-Neelain University**

# الآية

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

**قال تعالي:**

**(امَنَ الرَّسُولُ بِمَا أُنزِلَ إِلَيْهِ مِن رَّبِّهِ وَالْمُؤْمِنُونَ كُلٌّ آمَنَ بِاللَّـهِ وَمَلَائِكَتِهِ وَكُتُبِه وَرُسُلِهِ لَا نُفَرِّقُ بَيْنَ أَحَدٍ مِّن رُّسُلِهِ وَقَالُوا سَمِعْنَا وَأَطَعْنَا غُفْرَانَكَ رَبَّنَا وَإِلَيْكَ الْمَصِيرُ ﴿٢٨٥﴾ لَا يُكَلِّفُ اللَّـهُ نَفْسًا إِلَّا وُسْعَهَا لَهَا مَا كَسَبَتْ وَعَلَيْهَا مَا اكْتَسَبَتْ رَبَّنَا لَا تُؤَاخِذْنَا إِن نَّسِينَا أَوْ أَخْطَأْنَا رَبَّنَا وَلَا تَحْمِلْ عَلَيْنَا إِصْرًا كَمَا حَمَلْتَهُ عَلَى الَّذِينَ مِن قَبْلِنَا رَبَّنَا وَلَا تُحَمِّلْنَا مَا لَا طَاقَةَ لَنَا بِهِ وَاعْفُ عَنَّا وَاغْفِرْ لَنَا وَارْحَمْنَا أَنتَ مَوْلَانَا فَانصُرْنَا عَلَى الْقَوْمِ الْكَافِرِينَ﴿٢٨٦﴾**

صدق الله العظيم

الآيتين من سوره البقره (285-286)

## DEDICATION

For the sake of Allah, our creator and Master Mohammed, our greater teacher

To our dear parent for love, care and support

To our dear brothers and sisters, who stood beside us in all our

Life events

To my friends and colleagues for their support and encouragement.

# ACKNOWLEDGEMENT

Piously our gratitude and prayers to ALMIGHTY Allah for the mercy which followed us during the long path of this research. We more so match our supervisor ***DR. SHAR SAADELDAEN HAJMOHAMED***. For his close supervision, valuable advice and stimulating suggestions.

We sincerely appreciate her valuable advice, guidance and assistance in practical writing of dissertation.

We very much thank full to REEL JMAL as co-supervisor for tired with us to do this training period, and this valuable advice, guidance, can be interest and encouragement at various stage in our live.

We would thank and power full to our DR: OSAMA MOHAMMAD of gratitude's of "EXON" Molecular biology laboratory and patience of the staff of Napata college - Medical Laboratory Sciences Last but not least we thank anyone know us and who contributed by any means to this research form the commence, during the processing of the specimens to the final touches.

# ABSTRACT

**Background**: *Klebsiella pneumoniae (K. pneumoniae)* has become one of the most agents of healthcare-associated infection in medical centers with the potential to cause several clinical infections including pneumonia, septicemia, and urinary tract and soft tissue infections.

In recent years, the emergence of several types of resistance to multiple classes of antibiotics has caused critical clinical concern, leading to serious limitations in the treatment of these pathogens with subsequent increased morbidity and mortality.

The objective of this study was the detection of a resistance gene in *k.pneumoniae* isolate from different types of samples in hospitals (Alia Specialist, Yastabshroun, Military hospital, Shargalneel, Omdurman for maternity) in Khartoum state – Sudan.

A total of 50k.pneumoniae was isolated from different hospitals in Khartoum state.

**Objectives:** Detection of Aminoglycoside Resistance Gene (arm A, ant, Acc3, Acc6, and Aph) in *Klebsiella pneumoniae*.

**Method:** the cross-sectional analytical study was conducted in Khartoum state Sudan, A number of fifty samples were collected during the period from May to September 2022 and labeled culture these specimens in MacConkey agar in order to identify the bacteria perform gram staining technique and following biochemical tests (indole test, urease test, citrate utilization test, motility test, and growth on kligler iron agar) was performed and measure of antimicrobial sensitivity test by disc diffusion method (Kirby bour method), DNA extracted by use boiling method, and then perform multiplex PCR ( Ant andAcc3,Aph and Acc6 ) uniplex PCR (arm A).

**Result**: Out of the 50 isolates from different samples including Urine, swabs, blood, sputum, and aspiration, most isolates from wound swabs 27(54%) male to female ratio were 0.6:1, patients' ages ranged from 1 year to 78 years with mean 53 (SD=22), the result of aminoglycoside resistance showed 28(56%), 34 (68%) and 37(74%) Gentamicin, Amikacin, and tobramycin respectively. Acc3 gene were detected in 21(42%) and 21 (42%) arm A, followed by 6 (12%) positive for Ant, and Acc6 was found in two isolates (4%), Aph was not detected there is not isolate share two or more genes.

**Conclusion:** Our study findings confirmed the presence of aminoglycosides high resistance *Klebsiella pneumoniae* in Khartoum, Sudan with high prevalence of ant, armA, acc3 and acc6 genes especially acc3 and armA.

The correlation between age group in the <1 year (40.0%) and acc6 gene and association between blood sample (40.0%) and acc6 gene.

# الاطروحة

**الخلفية**: أصبح الالتهاب الرئوي*Klebsiella (K. pneumoniae)* أحدأكثرعوامل العدوى المرتبطة بالرعاية الصحية في المراكز الطبية مع إمكانية التسبب في العديد من العدوى السريرية بما في ذلك الالتهاب الرئوي وتسمم الدم والتهابات المسالك البولية والأنسجة المريضة.

في السنوات الأخيرة ، تسبب ظهور عدة أنواع من المقاومة لفئات متعددة من المضادات الحيوية في قلق سريري خطير ، مما أدى إلى قيود خطيرة في علاج هذه العوامل الممرضة مع زيادة معدلات المراضة والوفيات.

الهدف من هذه الدراسة هو الكشف عن جينات مقاومة في عزلة*k.pneumoniae*من أنواع مختلفة من العينات في المستشفيات (عليا التخصصي ,يستبشرن ,السلاح الطبي , شرق النيل , ام درمان للولادة ) في لاية الخرطوم - السودان.

تم عزل مجموعه 50 عينة من للكلبسيله الرئويه *k.pneumoniae* من مستشفيات مختلفة في ولاية الخرطوم.

**الأهداف:** الكشف عن الجين المقاوم للأمينوغليكوسايد (armA,acc3,acc6,ant,aph) في للكلبسيله الرئويه

الطريقة: أجريت الدراسة التحليلية المقطعية بولاية الخرطوم السودان ، حيث تم جمع في الفترة من شهر مايو الي شهر سبتمبر 2022 عدد خمسين عينة وزراعتها على هذا الوسط بأجارMacConkeyللتعرف على البكتيريا التي تؤدي تقنية صبغ الجرام وبعد الاختبارات الكيميائية الحيوية (اختبار إندول ، اختبار اليورياز) ، تم إجراء اختبار استخدام السترات ، واختبار الحركة ، والنمو على أجار الحديد (kliglerوقياس حساسية مضادات الميكروبات بطريقة الانتشار القرصي (طريقة كيربي بور) ، واستخراج الحمض النووي باستخدام طريقة الغليان ، ثم إجراء تفاعل البوليميراز المتسلسل المتعدد.

**النتيجة**: من بين 50 عينة من عينات مختلفة بما في ذلك البول والمسحات والدم والبلغم والطموح ، كانت معظم العزلات من مسحات الجرح 27 (54٪) نسبة الذكور إلى الإناث 0.6: 1 ، وتراوحت أعمار المرضى من سنة واحدة إلى 78 سنة بمتوسط 53 (SD = 22) ، أظهرت نتيجة مقاومة أمينوغليكوسايد 28 (56٪) ، 34 (68٪) و 37 (74٪) جنتاميسين ، أميكاسين ، وتوبراميسين على التوالي. تم الكشف عن جين Acc3 في 21 (42٪) و 21 (42٪) armA، تليها 6 (12٪) ant ، وتم العثور على Acc6 في عينتين (4٪) ، ولم يتم الكشف عن.Aph لم يوجد عينة بها مشاركة بين جينين او اكثر .

**الخلاصه:** أكدت نتائج دراستنا وجود الامينوغليكوسايد عالية المقاومة *klebsiella.pneumonae* في الخرطوم ، السودان مع انتشار مرتفع لجينات ant ، Aph، armA، acc3 و acc6 خاصةً acc3 و armA.

العلاقة بين الفئة العمرية في اقل من و جين ACC6 كانت النسبة(40%)و الانباط بين عينة الدم وجين ACC6ايضا بنسبة (40%).

## TABLES OF CONTENTS :

# LIST OF TABELS:

# Table of abbreviation

| | |
|---|---|
| MIC | Minimum inhibitory concentrations |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| ESBL | extended-spectrum β-lactamase |
| MDR | multidrug-resistant |
| XDR | extremely drug resistant |
| CLSI | Clinical and Laboratory Standards Institute |
| ACC | Aminoglycoside acetyltransferase |
| Ant | Aminoglycoside nucleotidyltransferase |
| Aph | Aminoglycoside phosphor transferase |
| Arm | Aminoglycoside ribosomal methylase |
| GN | Gentamicin |
| TOB | Tobramycin |
| AK | Amikacin |

# CHAPTER ONE

## INTRODUCTION

# 1. INTRODACION:

## 1.1 General characteristics:

Genus *Klebsiella* belongs to the tribe *klebsiellae,* a member of the family Enterobacteriaceae. The organisms are named after Edwin Klebs, a 19th century German microbiologist. *Klebsiella* spp. is nonmotile, straight rod shape, 0.3-1µm in diameter and 0.6-6µm in length, when these bacteria are stained with a Gram stain that gives pink color (negative bacteria) with a prominent polysaccharide capsule. [1]

*Klebeseilla* are non-motile members of the enterobacteriaceae, they are usually capsulate and can be recognized by their large, greyish-white mucoid colonies on laboratory medium, especially if it has high sugar content. They are phenylalanine deaminase negative, do not produce H2S in tsi agar or liquefy gelatin. Nearly all grow in kcn medium and ferment a wide range of carbohydrates including adonitol and inositol. The most frequently isolated *klebsiellae* ferment lactose,Most species hydrolyse urea but do so much less rapidly. Most are positive in the Voges –proskauer test, though strains from the respiratory tract often give atypical results in this and other tests. [2]

*Klebsiella* is present in the respiratory tract and feces of about 5% of normal individuals. It causes a small proportion (~1%) of bacterial pneumonias. *K pneumoniae* can produce extensive hemorrhagic necrotizing consolidation of the lung. It produces urinary tract infection and bacteremia with focal lesions in debilitated patients. Other enterics also may produce pneumonia. [3]

This organism grows well on ordinary nutrient media in a temperature range of 12 to 43°C with optimum growth at 37°C. Colonies are large, raised, moist and viscid which are typically designated as mucoid. The mucoid character is dependent upon the ability of bacterium to produce polysaccharide capsule as well as availability of polysaccharide in the medium. Since large number of strains ferment lactose, they give rise to pink coloured colonies.in addition The majority of k. *pneumoniae* were multi-drug resistant (82%).The bacteria were found resistant to most β-lactam antibiotics, aminoglycosides, ciprofloxacin, cotrimoxazole, carbapenem, piperacillin, and tazobactam. Howeve, only about 0.7% colistin resistance was observed [1] also, the antibiotic susceptibility of isolates was tested for six different classes of antibiotics: beta-lactams (imipenem, meropenem, cefotaxime, ceftazidime,piperacillintazobactam,aminoglycosides,gentamicin,amikacin,tobramycin),fluoro quinolone ,ciprofloxacintrimethoprim-sulfamethoxazole, tetracycline (tigecycline), and polymyxin B (colistin). The disk diffusion method was used according to the specifications of

the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Minimum inhibitory concentrations were determined using ETEST following manufacturer instructions. The plates were incubated at 37 °C for 18 h. EUCAST clinical breakpoint values for zone diameters (mm), and the MIC values were used to categorize the isolates as susceptible, intermediate, and resistant [4]

## 1.2 Normal Habitat:

Normal flora of human upper respiratory and genitourinary tract, the normal habitat of these bacteria is the intestinal tract of human and animal, but was causative agent causing a wide range of burn smears, wound smears, respiratory urinary tract infections and bacteremia. [3]

## 1.3 *Klebesiellae* species:

*Klebsiellae* species rank among the top ten bacterial pathogens responsible for hospital-acquired infections. Two other *Klebsielleae* are associated with inflammatory conditions of the upper respiratory tract: *Klebsiella pneumoniae* subspecies *ozaenae* has been isolated from the nasal mucosa in ozena, a fetid, progressive atrophy of mucous membranes; and *K pneumoniae* subspecies *rhinoscleromatis* form rhinoscleroma, a destructive granuloma of the nose and pharynx. *Klebsiella granulomatis* (formerly Calymmato bacterium granulomatis) causes a chronic genital ulcerative disease, granuloma inguinale, an uncommon sexually transmitted disease. The organism grows with difficulty on media containing egg yolk. Ampicillin or tetracycline is effective treatment disease. [3]

*Rhinoscleroma* and ozena are two clinical forms of chronic rhinitis and can present identical clinical pictures in their early stages, *Rhinoscleroma,* a chronic granulomatous disease of the upper airways, rarely occurs nowadays in Europe, where it was first described in 1870. However, it remains endemic in tropical and subtropical areas (North and Central Africa, Southeast Asia and Central and South America). Several predisposing factors such as living under conditions of poor hygiene and nutritional deficiencies have been described and are apparently necessary for transmission of the disease. With no gender preference, it more frequently affects young adults *Klebsiella rhinoscleromatis* is the etiologic agent of rhinoscleroma. [5]

*Ozena*, meaning ‘stench’, was first described in 1876. It is a rare, progressive, chronic rhinitis characterized by atrophic changes in the nasal mucosa with resorption of the underlying bone, formation of a thick and greenish crust, and a distinct fetid odor. The diagnosis is clinical: enlargement of the nasal passages and a foul-smelling mucopurulent discharge that tends to

dry into crusts. This disease, endemic in poor countries, is rarely isolated in developed countries because of better hygiene conditions. [5]

*Klebsiella granulomatis* (formerly Calymmato bacterium granulomatis) causes a chronic genital ulcerative disease, granuloma inguinale, an uncommon sexually transmitted disease. The organism grows with difficulty on media containing egg yolk. Ampicillin or tetracycline is effective treatment. [3]

*Klebsiella pneumoniae (K. pneumonia*) has become one of the most agents of health care associated infection in medical centers with the potential to cause several clinical infection including pneumonia, septicemia, urinary tract and soft tissue infections. In recent years, the emergence of several types of resistance to multiple classes of antibiotics has caused critical clinical concern, leading to serious limitation in the treatment of these pathogens with subsequent increased morbidity and mortality. [6]

## 1.4 Diseases and pathogeneses:

These organisms are usually opportunistic pathogens that cause nosocomial infections, especially pneumonia and urinary tract infections. *K. pneumoniae* is an important respiratory tract pathogen outside hospitals as well of the three organisms, *K. pneumoniae* is most likely to be a primary, non-opportunistic pathogen; this property is the exception of pneumonia caused by Klebsiella, which produces a thick, mucoid, bloody sputum ("currant-jelly" sputum) and can progress to necrosis and abscess formation. There are two other species of Klebsiella that cause unusual human infections rarely seen in the United States. Klebsiella ozaenae is associated with atrophic rhinitis, and *Klebsiella rhinoscleromatis* causes a destructive granuloma of the nose and pharynx, *Enterobacter* infections are clearly related to hospitalization, especially to invasive procedures such as intravenous catheterization, respiratory intubation, and urinary tract manipulations. In addition, outbreaks of pneumonia have been associated with contamination of the water in respiratory therapy devices. [3-6]

The virulence of *Klebsiellae* shows tremendous variation. Capsule does not seem to play an important part in causing the disease. The spectrum of diseases caused by this bacterium includes bronchopneumonia, wound sepsis, bacteraemia, and meningitis and urinary tract infections. Its role as an important pathogen in nosocomial infections is gaining recognition. [4]

## 1.5 Prevention:

Some hospital-acquired infections caused by gram-negative rods can be prevented by such general measures as changing the site of intravenous catheters, removing urinary catheters when they are no longer needed, and taking proper care of respiratory therapy devices. There is no vaccine. [7]

## 1.6 Treatment:

No single specific therapy is available. The sulfonamides, ampicillin, cephalosporins, fluoroquinolones, and aminoglycosides have marked antibacterial effects against the enteric, but variation in susceptibility is great, and laboratory tests for antibiotic susceptibility are essential. Multiple drug resistance is common and is under the control of transmissible plasmids. Certain conditions predisposing to infection by these organisms require surgical correction, such as relief of urinary tract obstruction, closure of a perforation in an abdominal organ, or resection of a bronchiectasis portion of lung. Treatment of gram-negative bacteremia and impending septic shock requires rapid institution of antimicrobial therapy, restoration of fluid and electrolyte balance, and treatment of disseminated intravascular coagulation. Various means have been proposed for the prevention of traveler's diarrhea, including daily ingestion of bismuth subsalicylate suspension and regular doses of tetracycline or other antimicrobial drugs for limited periods. Because none of these methods are entirely successful or lacking in adverse effects, it is widely recommended that caution be observed in regard to food and drink in areas where environmental sanitation is poor and that early and brief treatment. [3]

## 1.7 Antibiotic resistance in *Klebsiella pneumoniae:*

The overuse, underuse, and misuse of antibiotics have become major causes of the development of antibiotic resistance in bacteria also the emergence and spread of antibiotic-resistant bacteria has been a growing concern over the last decade, which can lead to serious clinical and public health problems. *Klebsiella pneumoniae*, an opportunistic pathogen, is mainly responsible for nosocomial infections with high morbidity and mortality moreover recently, the rapid emergence of extended-spectrum β-lactamase (ESBL) producing *K. pneumoniae* has significantly increased the risk of developing serious nosocomial and community-acquired infections worldwide also furthermore, multidrug-resistant *K. pneumoniae* strains can cause treatment failure with current antibiotic therapy and the proposed resistance mechanisms of *K. pneumoniae* against different classes of antibiotics

include release of antibiotic-inactivating enzymes, modification of antibiotic target sites, change in membrane permeability, activation of efflux pump systems, and alteration of metabolic pathways and Among these mechanisms, the enzymatic degradation and efflux pump systems play an important role in the development of multidrug resistance in *K. pneumoniae.* [8]

## 1.8 History of antibiotic resistance in *Klebsiella pneumoniae:*

After more than 70 years of extensive use of antibiotics to treat infectious diseases, antibiotic resistance is now being recognized as a worldwide crisis in modern medicine. The dramatic increase in prevalence of infections caused by multidrug-resistant (MDR) and extremely drug resistant (XDR) pathogens belonging to the *Enterobacteriaceae* group poses a great concern since these pathogens are common natural inhabitants of our micro biome. Moreover, infections caused by these strains are often associated with high mortality rates, prolonged hospitalization and costs. Antibiotic resistance is a multifactorial complex process. However, from the bacterial perspective it reflects evolution in action, concomitant to the continuous exposure to antibiotics, where selective pressure gives rise to the evolvement of multiple genetic mechanisms. This constant evolution over the years has led to the emergence of MDR and XDR *Enterobacteriaceae* strains that exhibit resistance to nearly all antibiotics available, without possible treatment option. The risk of global dissemination of these XDR pathogens has become a recognized global threat. In the context of antibiotic resistance, a 'successful' bacterial strain should be an extremely effective vehicle for the dissemination of antibiotic resistance traits. This can be accomplished if it can transfer its antibiotic resistance traits vertically to its daughter cells, and also if it can act as a donor for mobile genetic elements (MGEs), like plasmids and transposons, that may be transferred horizontally to other strains, species or age. In this era of antibiotic resistance, *Klebsiella pneumoniae* represents one of the most concerning pathogens involved in antibiotic resistance and as such, together with other highly important MDR pathogens, it has been classified as an ESKAPE organism. [9]

## 1.9 Aminoglycoside as general:

Aminoglycosides are a large family of drugs that act at the ribosome by inhibiting one or more of the biochemical steps involved in translation. They are extensively used in the treatment of serious bacterial infections, particularly in combination with β-lactams or glycopeptides. The increasing problem of multi resistance in Gram-negative bacteria and the introduction of new aminoglycoside analogues (e.g., plazomicin) warrant new studies aimed

at understanding aminoglycoside resistance. [10] The function of many antibiotics is based on the inhibition of a central biological process by binding of the drug to a specific RNA, Aminoglycoside antibiotics are intensively studied because they are widely used in therapy and are known to interact with a large variety of different RNA targets. Aminoglycoside antibiotics, used to treat persistent gram-negative infections, tuberculosis, and life-threatening infections in neonates and patients with cystic fibrosis, can infer acute kidney injury and irreversible hearing loss. [11] The full repertoire of cellular targets and processes leading to the toxicity of aminoglycosides is not fully resolved, making it challenging to devise rational directions to circumvent their adverse effects. As a result, there has been very limited effort to rationally address the issue of aminoglycoside-induced toxicity. Here we provide an overview of the reported effects of aminoglycosides on cells of the inner ear and on kidney tubular epithelial cells. We describe selected examples for structure–toxicity relationships established by evaluation of both natural and semisynthetic aminoglycosides. The various assays and models used to evaluate these antibiotics and recent progress in development of safer aminoglycoside antibiotics are discussed. [12]

### 1.9.1 Toxicity of aminoglycosides:

The significant clinical toxicities of aminoglycosides are ototoxicity, nephrotoxicity and less often neuromuscular toxicity. There is no definitive evidence of differences in the degree of toxicity among the three commonly used agents (gentamicin, tobramycin and amikacin). Patient factors that vary the risk of toxicity include pre-existing disease, severity of illness, concomitant drugs administered and genetic predisposition. In addition, prolonged therapy of aminoglycosides has shown to be an independent risk factor for toxicity. Nephrotoxicity occurs after glomerular filtration of the agent in the proximal convoluted tubule. Ototoxicity, including vestibular and cochlear toxicity, occurs from damage to the sensory hair cells of these organs. The exact pathophysiological mechanism at both sites is incompletely understood. Neuromuscular blockade after administration is described, usually in conjunction with other diseases or drugs that affect the neuromuscular junction, for example, patients with myasthenia gravis. Although a genetic susceptibility mutation has been reported (mitochondrial 1555A→G) in patients with kindred's with an inherited susceptibility to hearing loss, the population prevalence of these mutations (1:500) is probably too infrequent to account for most cases of ototoxicity. The incidence of vestibular or cochlear toxicity varies in studies with the definition used, and most were performed before the era of high-dose-extended interval regimens. However, most reviews have

reported rates of approximately 5–10% for hearing impairment and approximately 3% for vestibular toxicity. The rate of ototoxicity with durations of treatment less than 48 h is not known; however, ototoxicity following single doses has been described. Monitoring for toxicity can be through three mechanisms; quantitative testing of end-organ effects (monitoring serum creatinine and audiometry), active bedside testing and passive reporting by the patient. There is no definitive evidence to inform optimal techniques for monitoring of toxicity. As a minimum standard, before the commencement of aminoglycoside therapy patients should be informed about the possible adverse effects and asked to report if they develop subjective hearing loss, tinnitus, and serum creatinine should be monitored in all patients. Where the expected duration of therapy is more than 5 days, bedside tests should be performed for cochlear and vestibular function. The 'whisper test' has been shown to have a high sensitivity for hearing impairment within the clinically relevant frequency range, but does not detect high-frequency hearing loss that often occurs earlier. Additionally, hearing loss may occur after cessation of the antibiotic course. Where available, serial audiometry (pure tone audiometry or otoacoustic emissions) may be considered; the development of high-tone hearing impairment and impaired outer cell function is characteristic of drug-induced damage. Diagnosis is most accurate if a baseline result is available for comparison, as similar changes may be found in patients with hearing loss because of other causes, including presbycusis (age-related hearing loss). There is no definitive evidence that ceasing aminoglycosides when ototoxicity is detected minimizes further damage, but it would seem prudent to weigh up the benefits of continuing with the significant risk of ototoxicity in this situation. [13]

### 1.9.2 Aminoglycoside resistance:

Aminoglycoside resistance in Gram-negative bacteria can be endogenous or acquired. Examples of clinically encountered bacteria exhibiting endogenous resistance include *Stenotrophomonas maltophilia* and *Burkholderia cepacia*. Acquired resistance can occur in almost all Gram-negative organisms, including commonly encountered bacteria, such as *Klebsiella pneumoniae*. Acquired resistance is frequently encoded by genes located on a transferrable plasmid, increasing the likelihood of horizontal spread to other bacteria. It is frequently associated with other resistance genes, such as those encoding extended spectrum β-lactamases, rendering bacteria resistant to multiple classes of antibiotics. There is a wide variety of mechanisms of aminoglycoside resistance. The most common is enzymatic inactivation of the aminoglycoside through acetylation, acetylation or phosphorylation. Other

less frequent mechanisms include decreased antimicrobial permeability, active efflux pumps and various changes in the ribosomal target site. A newly described mechanism of resistance is methylation of 16S ribosomal RNA. This mechanism results in high level resistance to gentamicin, tobramycin and amikacin. In the drug development pipeline are next-generation aminoglycosides or 'neoglycosides', synthesized by extensive modification of current agents. They have demonstrated promising *in vitro* activity against bacteria resistant to currently available aminoglycosides. However, they are unlikely to be active against strains which produce 16S ribosomal RNA methylases. [14]Therapy with combinations of a cell wall–active antibiotic (a penicillin or vancomycin) plus an aminoglycoside (streptomycin or gentamicin) is essential for severe enterococcus infections, such as endocarditis. Although enterococci have intrinsic low-level resistance to aminoglycosides (MICs <500 μg/mL), they have synergistic susceptibility when treated with a cell wall–active antibiotic plus an aminoglycoside However, some enterococci have high-level resistance to aminoglycosides (MICs >500 μg/mL) and are not susceptible to the synergism. This high-level aminoglycoside resistance is due to enterococcus aminoglycoside-modifying enzymes. The genes that code for most of these enzymes are usually on conjugative plasmids or transposons. The enzymes have differential activity against the aminoglycosides. Resistance to gentamicin predicts resistance to the other aminoglycosides except streptomycin. (Susceptibility to gentamicin does not predict susceptibility to other aminoglycosides.) Resistance to streptomycin does not predict resistance to other aminoglycosides. The result is that only streptomycin or gentamicin (or both or neither) is likely to show synergistic activity with a cell wall–active antibiotic against enterococci. Enterococci from severe infections should have susceptibility tests for high-level aminoglycoside resistance (MICs >500 μg/mL for gentamicin and >1000 μg/mL for streptomycin in broth media) to predict therapeutic efficacy. [3]Resistance to Aminoglycosides Analogous to beta-lactam resistance, aminoglycoside resistance is accomplished by enzymatic, altered target, or decreased uptake pathways Gram positive and gram-negative bacteria produce several different aminoglycoside-modifying enzymes. Three general types of enzymes catalyze one of the following modifications of an aminoglycoside molecule Phosphorylation of hydroxyl groups Acetylation of hydroxyl groups Acetylation of amine groups also once an aminoglycoside has been modified its affinity for binding to the 30S ribosomal subunit may be sufficiently diminished or totally lost, allowing protein synthesis to occur. Aminoglycosides enter the gram-negative cell by passing through outer membrane porin channels. Therefore, poring alterations may also contribute to aminoglycoside resistance among these bacteria. Although

some mutations that resulted in altered ribosomal targets have been described, this mechanism of resistance is rare in bacteria exposed to commonly used aminoglycosides. [15]

### 1.9.3 Aminoglycosides Resistance Mechanism:

Bacteria resistance to various self-method and acquired mechanisms, because they offer high levels and broad-spectrum resistance to aminoglycosides by covalent attaching a CH3 group to certain sites within the 16S rRNA a site. High-level aminoglycoside resistance is likely as a result of some aminoglycosides' projected significantly decreased binding affinity to the CH3-added 16S rRNA compared to that of the original 16S rRNA. The Aminoglycoside is 2 DOS core primarily to bind to 44 helix of the 16SrRNA share with bacteria 30Sribosomal sub unit. The aminoglycoside is distribution in protein synthesis and binding causes various, the distributing transfer RNA (tRNA) translocation to decrease translocation fidelity. Mobility and the ribosomal sub unit interfering. The study of the result recent information regarding the 16SRTMase gene responsible to resistance of aminoglycoside isolate in roundel in Korean community setting. ArmA were the predominate16SRMTase gene and the armA is worldwide distributed the further studies with a big number of clinical isolate are needed to confirm the high presence of vrarity of 16SRMTase according to aminoglycoside resistance of bacteria in Korea. [16]

### 1.9.4 Mechanism of Aminoglycosides Resistance of Enzyme:

Aminoglycosides are the mother of the treatment and key of department of antimicrobial for kill or inhibit gram negative bacterial. The aminoglycoside bind is highly conserved in the A site of the bacterial 30S ribosomal subunit's 16 SrRNA. Exogenously acquired 16S ribosomal RNA methyltransferases (16S RMTases) have emerged as a major mechanism of high-level resistance to the majority of clinically important aminoglycosides, including arbekacin, amikacin, tobramycin, and gentamicin in Gram-negative pathogens, despite the fact that several pathways that provide resistance to aminoglycoside antibiotics are known. The first acquired 16S RMTase genes, armA and rmtA, were discovered in *Klebsiella pneumoniae and Pseudomonas aeruginosa* in 2003. Another study discovered the gene arnA in *k.pneumoniae* in plasmid pil204 in2000. Since, other amplicon 16S RMTase genes have been discovered in clinical isolates (rmtB through rmtH, and npmA). The genes armA and rmtB have been found in several species of Gram-negative bacilli in Asia. Amikacin resistance rates for *K. pneumoniae* grew from 8% in 1997 to 13% in 2003 according to a national surveillance in Korea. For *Enterobacter cloacae* and *Citrobacter*, the occurrence of high-level resistance to amikacin or arbekacin was 9.5% (15/158), 10.3% (13/126), and 17.1% (22/129) isolates of

Serratia marcescens and freundii, respectively the further investigation whether resistance of aminoglycoside with transferable the resistance of plasmid from clinical isolate with high level to amikacin (<256ug\ml). [17]

### 1.9.5 Literature Review:

#### 1.9.5.1 Previous studies in aminoglycosides resistant in Sudan:

There were many studies that conducted in Sudan in aminoglycosides resistance in gram negative bacteria including *Klebsiella pneumoniae*. In 2008 a large study that conducted in 166 isolates from different clinical specimens, 199 were *klebsiella.pneumoniae* tested for their susceptibility to different antimicrobial drugs included aminoglycosides showed that 86.6%, 77.7% and 91% for Pipracillin, Tobramycin and Ampicillin respectively. [18] and this was a high resistance when compare with another one in eight hospitals in Khartoum statein 2016 , a total of 734 Gram-negative bacilli were isolated from to detect a multidrug resistance bacteria, 249 (34%) isolates were confirmed as *Klebsiella pneumoniae* as the most frequently encountered one, aminoglycosides show low resistance as showed 18.3% , 52.5% resistant to Amikacin and Gentamycin respectively. [19]

In research that conducted in Soba University Hospital over six months period in 2018 a total of 734 Gram-negative bacilli were isolated *Klebsiella spp.* were 249 (34%), in the analysis of the antimicrobial susceptibility patterns showed that 134 (22.3%) of total gram negative isolates were a multidrug resistant to three or more classes of antibiotics including aminoglycosides. [20] Another Sudanese research publicated in 2021 in UTI patients a total of 70 urine samples were collected, 7% were *K. pneumoniae* in order to detect aminoglycosides resistant and 16% isolates were resist to it ( 60% to Tobramycin, 0% to Kanamycin, 20% to Streptomycin, 0% to Gentamycin, and 0% to Amikacin.[21]In 2019, 300 Isolates of Klebsiella spp were collected from different hospitals in Khartoum state, from 79 MDR isolates 63 (79.7%)were resist to Gentamicin. [22]

#### 1.9.5.2 Previous studies in aminoglycosides resistance genes around the world:

In 2013 this study, were developed a GeXP analyzer-based multiplex PCR assay to simultaneously detect seven aminoglycoside-resistance genes, including *aac(3)-II, aac(6')-*Ib, *aac(6')-II, ant(3")-I,aph(3')-VI,armA* and *rmtB,* and to analyze the distribution of these genes in clinical *Enterobacteriaceae* isolates. Under optimized conditions, this assay achieved a limit-of-detection as low as 10 copies of each of the seven genes, in 56 clinical Enterobacteriaceae isolates; the results were compared with that of the conventional single PCR assay. Kappa values of the two methods for detecting each of the seven resistance genes were 0.831, 0.846, 0.810, 0.909, 0.887, 0.810 and 0.825, respectively. [23] Later on new study conducted in 2017 a number of 307 *Enterobacteriaceae* isolates were collected from

five hospitals in northwest Iran. The isolates were identified as *E. coli* (219 isolates), *Klebsiella pneumoniae* (57 isolates), *Enterobacter cloacae* (14 isolates), *Proteus mirabilis* (5 isolates),*Klebsiella oxytoca* (2 isolates), *Proteus vulgaris* (2 isolates), *Morganellamorganii* (2 isolates),*Shigellasonnei* (2 isolates), *Shigellaflexneri* (2 isolates), *Citrobacterfreundii* (1 isolate), and *Serratiamarcescens* (1 isolate). The disk diffusion method for amikacin, gentamicin, tobramycin, kanamycin, and streptomycin, as well as the minimum inhibitory concentration for amikacin, gentamicin, tobramycin, and kanamycin were done for susceptibility testing. Overall, 220 isolates (71.7%) were resistant to at least one aminoglycoside antibiotic, Amikacin showed the highest susceptibility rate (93.8%) followed by kanamycin (66.1%), gentamicin (63.5%), tobramycin (60.6%), and streptomycin (47.6%).Thirteen AME genes and *armA* methylase were screened using the PCR and sequencing assays.Two hundred and twenty (71.7%) of isolates were resistant to aminoglycosides and 155 (70.5%) of them were positive for aminoglycoside resistance genes. The most prevalent AME genes were *ant (3")-Ia* and *aph (3")-Ib* with the frequency 35.9% and 30.5%, respectively.[23] In the same year anther study conducted Investigation of genes encoding AMEs revealed that *acc(6')-Ib* was the most prevalent, followed by *acc(3')-II, aph(3')-IV,* and *ant(3")-I*. Examination of genesFrom 301 recovered isolates, 114 (37%) were *K. pneumoniae,* 3 (1%) were *Klebsiella oxytoca,* 61 (20%) were *E. coli*, 48 (16%) were *Proteus* spp., 38 (13%) were *Pseudomonas aeruginosa*, 12 (4%) were *Acinetobacterbaumannii,* 9 (3%) were *Serratiamarcescens,* 6 (2%) were *Enterobacter cloacae,* and 3 (1%) were single isolates for each of *Providenciastuartii, Burkholderia cepacia,* and *Aeromonashydrophilia*. All *K. pneumoniae* isolates were resistant or intermediate-resistant isolates to either gentamicin or amikacin. [24] Among the original 158 CR-Kp isolates, 91.77% (145/158) had at least one clinically relevant aminoglycoside-resistanceAs a group, 99.37%, 84.81%, 82.28% and 10.76% of the CR-Kp isolates were susceptible to plazomicin, amikacin, gentamicin and tobramycin, respectively. 72.23% of the total variance in aminoglycoside MICs and separated isolates into four groups with *aac(6')-Ib, aac(6')-Ib', aac(6')-Ib+aac(6')-Ib'* or no clinically relevant aminoglycoside-resistance genes, Within the validation cohort, the categorical agreement when comparing the observed BMD MICs with the predicated MICs was 96.55%, 89.66%, 86.21% and 82.76% for plazomicin, gentamicin, amikacin and tobramycin, respectively. [25] The newest study conducted in 2019 out of 181 MDR bacteria genotyped, 69(38.12%) tested positive for at least one of the genotyped AMGs. Highest (50, 27.62%) detected gene was ant (3″) $^{c}$ followed by aph (3″)$^{c}$(33, 18.23%). Combination of aph (3″)$^{c}$ and ant (3″)$^{b}$ in a single

bacteria was observed as the highest (14, 7.73%) among the detected gene combination. Alcaligenessp showed the highest (10/20) occurrence of ant $(3'')^{b}$ while aph $(3'')^{c}$ was the highest detected among Proteus sp (11/22). Other bacteria that showed the presence of AMGs include: Acinetobacter, Aeromonas, Bordetella, Brevundimonas, Chromobacterium, Klebsiella, Leucobacter, Morganella, Pantoae, Proteus, Providencia, Psychrobacter and Serratia. [26]

### 1.9.6 Rational:

This research will identify *Klebsiella pneumoniae* that are resistant to aminoglycosides, which are among the bacteria that are frequently found in hospitals and cause severe infections affected by Overpopulation during community unfortunately, the country did not show data, recently the word has been complaining about bacteria that are resistant to antibiotics, which causes problems when taking drugs.

Antimicrobial-resistant infections may cause significant threat to the public health systems leading to the high rates-of morbidity and mortality, and may contribute to raped rising of global healthcare costs resistance to antibiotics occurs when bacteria become able to adapt and grow in the presence of antibiotics that designed to kill them.

The isolate is susceptible to antibiotic action. Despite the fact that "took the place of colistin in the 1970s because they were thought to be" less hazardous new research suggests that colistin may actually be less dangerous than aminoglycosides.

### 1.9.7 Objectives:

### 1.9.7.1 General objective:

Detection of Aminoglycoside Resistance Gene arm A, ant, Acc3, Acc6 and Aph in *Klebsiella pneumoniae*.

### 1.9.7.2 Specific objective:

-Re-identify isolated bacteria by use stander culturing and biochemical technique

-To determine resistance profile to aminoglycosides (AK, CN, TOB).

-To detect aminoglycosides resistances genes, arm A, ant, Acc3, Acc6 and Aph in *Klebsiella pneumoniae* by use multiplex and uniplex PCR.

-To determine association between antimicrobial susceptibility and demographic variable.

-To determine association between present of aminoglycosides resistances genes and demographic variable.

# CHAPTER TWO

## MATERIALS AND METHODS

# 2. MATERIAL AND METHOD:

## 2.1 study design:

This study was crossed-sectional analytical laboratory based study.

## 2.2 study area:

This study was conducted in hospitals (Alia Specialist, Yastabshroun, Military hospital, Shargalneel, Omdurman for maternity) in Khartoum State-Sudan

## 2.3 study duration:

This study was conducted during the period from May to September 2022.

## 2.4 study sample:

The isolate *k.pneumoniae* was obtained from hospitals which were previously isolate from different clinical specimens. (Urine, Wound, Swabs, Blood, Body fluids)

## 2.5 sample size:

A total of Fifty (n=50) *k.Pneumoniae* clinical isolate were collected for this study.

## 2.5.1 Inclusion criteria:

*k.Pneumoniae* that resistance to aminoglycoside.

## 2.5.2 Exclusion criteria:

*K. pneumoniae* isolate that sensitive to aminoglycoside.

## 2.6 data collection:

Structured questionnaires were used to collect data from hospital questionnaire is created by researcher and consist of (type of sample, age, gander). Of patients were collected from hospitals medical records check list.

## 2.7 Ethical clearance:

The study was approved by the Ethical board of Napata College Khartoum (Sudan).

## 2.8 laboratory test:

## 2.8.1 Sample purification and identification:

Colony from each isolate were cultured on MaCconkey agar, tryptophan peptone water, semi-solid media, simmon citrate agar, christansis urea agar and kliglar iron agar, which incubated aerobically at 37°C overnight.

**2.8.2 Colonial morphology:** Most colonies show medium in size, flat, round; opaque, mucoid, pink lactose ferment and deamination give yellow color colonies.

### 2.8.3 Indirect gram stain:

Thin smears were prepared from overnight culture on clean slide and then fixed by heat.

Then made gram staining technique by add Crystal violet stain for 60 Sec, wash by water, add laugas's iodine for 60 Sec, then washed by tap water, smear was decolorized by Acetone-Alcohol rapidly for 7 Sec washed by tap water and finally covered by safranin for 2min and finally washed by tap water, then let the smear dry by air, drop of oil add to dried smear and examine by light microscope by use X100.

Which show gram negative short bacilli

### 2.8.4 Indole test:

In 1 ml tryptophan peptone water inoculated by use sterile wire loop, incubated aerobically at 37°C overnight, in next day add few drops of Kovac's reagents (4 (p) dimethylaminopenz aldehyde). No red ring was appearing.

### 2.8.5 Motility test:

In sterile semi solid media inoculated by use sterile straight loop. Incubated aerobically at 37°C overnight, in next day which no diffusion appears.

### 2.8.6 Citrate utilization test:

In simmon citrate agar by use sterile straight loop. Incubated aerobically at 37°C overnight, in next day which color change to blue.

### 2.8.7 Urease test:

In Christans urea agar by use sterile straight loop. Incubated aerobically at 37°C overnight, in next day which color change to pink.

### 2.8.8 Kliglar iron agar test:

In kliglar iron agar by use sterile straight loop. Incubated aerobically at 37°C overnight, in next day which color change to yellow butt yellow slop, gas without $H_2S$ production of the media and the bacteria give rad color in the tube is deamination.

### 2.8.9 Susceptibility test:

All isolates were tested on 50 Muller-Hinton agar plates (pH 7.2-7.4), the surface was inoculated lightly and uniformly by a sterile cotton swab, Before inoculation, the cotton swab was dipped into bacterial suspension with visually equivalent turbidity to 0.5% McFarland standards, the swab was then taking out and squeezed into the test tube wall to discard extra

fluid, the antimicrobial discs were placed and distributed evenly using sterile forceps on the inoculated plate. After aerobic incubation at 37˚C overnight inhibition zones were measured in millimeters (mm) using a ruler over the surface of the MH agar plate and the zones' diameters were recorded and interpreted according to CLSI guidelines 2022 as susceptible.

## 2.9 Molecular Detection of arm A, ant, Acc3, Acc6 and Aph gene

### 2.9.1 DNA extraction:

Bacterial genomic DNA was extracted by boiling method in biosafety cabinet level ıı from fresh overnight incubated nutrient slope, aloopful of culture was suspended in 200 µl of D.W sterile in 1.5µl eppendorf tubes heating for 10 min at 95°C in thermal block incubator, vortex, then cooling at -20°C for 10 min following by centrifuged for 10 min at 12000rpm , the supernatant was carefully collected and store at -20°C until use, all samples were checked for quantity using pre-PCR gel electrophoresis. [27]

### 2.9.2 PCR Primers for identification of arm A, ant, Acc3, Acc6 and Aph gene:

Table2-1: Primers sequence used for amplification of arm A, ant, Acc3, Acc6 and Aph gene:

| Target gene | Sequence(5'- 3') | Ampliction size (bp) | Reference |
|---|---|---|---|
| **Acc(3)** | **Fw:**CGGAAGGCAATAACGGAG<br>**Rev:**TCGAACAGGTAGCACTGAG | **740** | **[28]** |
| **Aac6** | **Fw:**TTGCGATGCTCTATGAGTGGCTA<br>**Rev:** CTCGAATGCCTGGCGTGTTT | **369** | **[29]** |
| **Aph** | **Fw:** GGCTAAAATGAGAATATCACCGG<br>**Rev:** CTTTAAAAAATCATACAGCTCGCG | **523** | **[30]** |
| **Ant** | **Fw:** CATCATGAGGGAAGCGGTG<br>**Rev:** GACTACCTTGGTGATCTCG | **800** | **[29]** |
| **armA** | **Fw:** CCGAAATGACAGTTCCTATC<br>**Rev:** GAAAATGAGTGCCTTGGAGG | **846** | **[29]** |

### 2.9.3 Preparation of primers:

The primer stock was made by adding 300 µL of sterile nuclease-free water to the forward primer and 320 µL to the reverse primer, and then 100 µL was made by putting 10 µL of each in 90 µL of sterile nuclease-free water in a fresh 1.5 Eppendorf tube, vorticing, and storing at -20oC until use.

### 2.9.4 Master Mix:

Master Mix kits (PCR PreMix, Korea) containing all reagents for PCR except water, template and primers were used and storage at (-20˚C). PCR was carried out in a 25 µl volume for detection of arm A, ant, Acc3, Acc6 and Aph genes in the following volumes in 0.2 ml Eppendorf tube.

**Table 2.2: Preparation of Reaction Mixture for arm A, ant, Acc3, Acc6 and Aph genes amplification:**

| | |
|---|---|
| Premix | 5 µl |
| Nuclease-free water | 13µl |
| Primers mixture | 2 µl |
| DNA template | 5 µl |
| Total volume | 25 µl |

### 2.9.5. Table (2.3) Protocol used for amplification of the armA and ant, Acc3 and Acc6, Aph genes:

| Gene | Typeofpcr | Name of Machine | Protocol | R |
|---|---|---|---|---|
| **arm A** | Uniplex PCR | Sens Quest | Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 5 minutes, and one cycle of final elongation at 72°C | **26** |
| **Ant and Acc3** | Multiplex PCR | Bio-Rad c1000 Touch | Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 5 minutes, and one cycle of final elongation at 72°C. | **26** |
| **Aph and Acc6** | Multiplex PCR | Bio-Rad c1000 Touch | Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, 55∘ C for 1 min, and 72°C for 5 minutes, and one cycle of final elongation at 72°C | **26** |

### 2.9.6 Electrophoresis of the Samples:

The gel casting tray was flooded by 1X TAE buffer near the gel cover surface, 5µl of PCR products of each sample was loaded into each well, then to the first and last wells of casting tray (3 µl) of (100bp) DNA ladder (marker) was injected for each run (Solis BioDyne, Europe). The gel electrophoresis apparatus was connected to the power supply (JY600; Beijing Junji-Dongfang, China). Then the electrophoresis was run at (120) V for (120 minutes)

### 2.9.7 Visualization of PCR product:

The gel was visualized under the ultraviolet Trans- illuminator (Bio-Rad, UK), to detect the specific amplified products by comparing with 100 base pairs standard ladders.

### 2.9.8 Analysis of data:

Analysis of data, Statistical Package for Social Sciences software, version 23.0 (IBM SPSSInc., Chicago, IL) was used. Initially, all information gathered via data collection sheet then coded into variables. Both descriptive and inferential statistics involving Fisher's exact Test were used to present the results.

# CHAPTER THREE

## RESULTS

# 3. RSULTS:

## 3.1 Demographic data:

A cross sectional analytical study was conducted at Khartoum state - during the period from August 2022 to October 2022.Fifteyisolates of k.*pneumoniae* were collected from different hospitals (Alia Specialist, Yastabshroun, Military hospital, Shargalneel, Omdurman for maternity).

Out of the 50 isolates, 15 (30%) were isolated from urine samples, 27 (54%) from wound swabs, 5 (10%) from blood, 1 (2%) from sputum, 2 (4%) from Aspiration.Which show on table (3.1).

Demographic data show out of 50 the male 19 were (38%) where female were 31 (62%) ages ranged from 5(10%) Less than 1 year, 6(12%) 20 to 40 year, 18(36%) 41 to 60 year, 21(42%) more than 60 years. Show on table (3.2).

Out of 50 k.*Pneumoniae* isolates only most isolates were resisting to all antibiotics, Approximately of isolates were resistant to 28(56%) resistant to Gentamicin,34(68%) resistant to Amikacin, and 37(74%) to resistant tobramycin, table (3.3, 3.4, 3.5)show the association between antibiotics susceptibility pattern and demographic data (age, gender, type of sample), and there is a relationship between tobramycin antibiotic and age group (*p*.value<0.05).

Out of fifty samples, Acc3 gene were detect in 21 (42%), and arm A show similar results 21 (42%), following by 6 (12%) positive for Ant, Acc6 was found in two isolates (4%), andAph were not detected. This showed on table (3.6).

Table (3.7) show the association between demographic data and genes the significant only on age group and blood sample and present of Acc6 gene (*p*.value 0.00 and 0.001 respectively)

**Table (3.1): Type of samples:**

| Type of sample | Number | Percentage |
|---|---|---|
| Sputum | 1 | 2% |
| Aspiration | 2 | 4% |
| Blood | 5 | 10% |
| Urine | 15 | 30% |
| Wound swab | 27 | 54% |

**Table (3.2): Distribution of gender among different age groups:**

| | | Gender | | Total |
|---|---|---|---|---|
| | | Male | Female | |
| Age group | <1 | 2(4%) | 3(6%) | 5(10%) |
| | 20-40 | 3(6%) | 4(8%) | 7(14%) |
| | 40-60 | 5(10%) | 12(24%) | 17(34%) |
| | >61 | 9(18%) | 12(24%) | 21(42%) |
| Total | | 19(38%) | 31(62%) | 50(100%) |

**Table (3.3): association between Demographic data and AK antibiotic:**

| Variables | | AK | | |
|---|---|---|---|---|
| | | **Sensitive** | **Resistant** | **Fisher's Exact Test P value** |
| **Gender** | **Male** | 3 | 13 | **0.744*** |
| | | 15.80% | 68.40% | |
| | **Female** | 7 | 21 | |
| | | 22.60% | 67.70% | |
| **Age groups** | **Less than 1 years** | 1 | 4 | **0.723*** |
| | | 20.00% | 80.00% | |
| | **20-40 years** | 0 | 4 | |
| | | 0.00% | 66.70% | |
| | **41-60 years** | 4 | 12 | |
| | | 22.20% | 66.70% | |
| | **More than 60 years** | 5 | 14 | |
| | | 23.80% | 66.70% | |
| **Type of sample** | **Urine** | 3 | 10 | **0.999*** |
| | | 20.00% | 66.70% | |
| | **Wound swab** | 6 | 17 | |
| | | 22.20% | 63.00% | |
| | **Blood** | 1 | 4 | |
| | | 20.00% | 80.00% | |
| | **Aspiration** | 0 | 2 | |
| | | 0.00% | 100.00% | |
| | **Sputum** | 0 | 1 | |
| | | 0.00% | 100.00% | |

**Table (3.4): association between Demographic data and GN antibiotic:**

| Variables | | GN | | |
|---|---|---|---|---|
| | | Sensitive | Resistant | Fisher's Exact Test P value |
| Gender | Male | 9 | 8 | 0.223* |
| | | 47.40% | 42.10% | |
| | Female | 7 | 20 | |
| | | 22.60% | 64.50% | |
| Age groups | Less than 1 years | 1 | 2 | 0.060* |
| | | 20.00% | 40.00% | |
| | 20-40 years | 5 | 1 | |
| | | 83.30% | 16.70% | |
| | 41-60 years | 6 | 10 | |
| | | 33.30% | 55.60% | |
| | More than 60 years | 4 | 15 | |
| | | 19.00% | 71.40% | |
| Type of sample | Urine | 5 | 10 | 0.327* |
| | | 33.30% | 66.70% | |
| | Wound swab | 8 | 15 | |
| | | 29.60% | 55.60% | |
| | Blood | 1 | 2 | |
| | | 20.00% | 40.00% | |
| | Aspiration | 1 | 1 | |
| | | 50.00% | 50.00% | |
| | Sputum | 1 | 0 | |
| | | 100.00% | 0.00% | |

**Table (3.5): association between Demographic data and TOB antibiotic:**

| Variables | | TOB | | |
|---|---|---|---|---|
| | | Sensitive | Resistant | **Fisher's Exact Test P value** |
| **Gender** | **Male** | 3<br>15.80% | 14<br>73.70% | |
| | **Female** | 5<br>16.10% | 23<br>74.20% | **0.999*** |
| **Age groups** | **Less than 1 years** | 4<br>80.00% | 1<br>20.00% | |
| | **20-40 years** | 1<br>16.70% | 4<br>66.70% | |
| | **41-60 years** | 0<br>0.00% | 17<br>94.40% | |
| | **More than 60 years** | 3<br>14.30% | 15<br>71.40% | **0.005**** |
| **Type of sample** | **Urine** | 3<br>20.00% | 10<br>66.70% | |
| | **Wound swab** | 1<br>3.70% | 23<br>85.20% | |
| | **Blood** | 4<br>80.00% | 1<br>20.00% | |
| | **Aspiration** | 0<br>0.00% | 2<br>100.00% | |
| | **Sputum** | 0<br>0.00% | 1<br>100.00% | **0.017**** |

- *. P value >0.05that's considered as statistically insignificant.
- **. P value <0.05that's considered as statistically significant.

**Table (3.6): show arm A, ant, Acc3, Acc6 and Aph genes among *Klebsiella pneumoniae*isolates:**

| **Resistant genes** | **Present** | |
|---|---|---|
| **ANT** | 6 | 12% |
| **ACC3** | 21 | 42% |
| **APH** | 0 | 0% |
| **ACC6** | 2 | 4% |
| **ARMA** | 21 | 42% |

**Table (3.7): Association between demographic data and presence of aminoglycoside Resistant gene:**

| | | | ACC6 | ANT | Arm A | Acc3 |
|---|---|---|---|---|---|---|
| Age groups | <1 | Count | 2 | 0 | 2 | 1 |
| | | % within age | 40.0% | 0.0% | 40.0% | 20.0% |
| | 20-40 | Count | 0 | 1 | 4 | 2 |
| | | % within age | 0.0% | 14.3% | 57.1% | 28.6% |
| | 40-60 | Count | 0 | 4 | 5 | 8 |
| | | % within age | 0.0% | 23.5% | 29.4% | 47.1% |
| | >60 | Count | 0 | 1 | 10 | 10 |
| | | % within age | 0.0% | 4.8% | 47.6% | 47.6% |
| Total | | Count | 2 | 6 | 21 | 21 |
| | | % within age | 4.0% | 12.0% | 42.0% | 42.0% |
| ***P*- value** | | | 0.00 | 0.27 | 0.56 | 0.58 |
| Gender | Male | Count | 1 | 3 | 7 | 8 |
| | | % within gender | 5.3% | 15.8% | 36.8% | 42.1% |
| | Female | Count | 1 | 3 | 14 | 13 |
| | | % within Gender | 3.2% | 9.7% | 45.2% | 41.9% |
| Total | | Count | 2 | 6 | 21 | 21 |
| | | % within Gender | 4.0% | 12.0% | 42.0% | 42.0% |
| ***P*-value** | | | 1.00 | 0.66 | 0.76 | 1.00 |
| Sample types | Urine | *Count* | 0 | 2 | 5 | 8 |
| | | *% within sample* | 0.0% | 13.3% | 33.3% | 53.3% |
| | Wound swab | *Count* | 0 | 4 | 12 | 11 |
| | | *% within sample* | 0.0% | 14.8% | 44% | 40.7% |
| | Blood | *Count* | 2 | 0 | 2 | 1 |
| | | *% within Sample* | 40.0 % | 0.0% | 40% | 20% |
| | Aspiration | *Count* | 0 | 0 | 2 | 0 |
| | | *% within sample* | 0.0% | 0.0% | 100% | 0.0% |
| | Sputum | *Count* | 0 | 0 | 0 | 1 |
| | | *% within sample* | 0.0% | 0.0% | 0.0% | 100% |
| ***P*- value** | | | 0.001 | 0.85 | 0.40 | 0.32 |

# CHAPTER FOUR

## DISCUSSION, CONCLUTION AND RECOMMENDATIONS

.

# 4. DISCUSSION CONCLUTION AND RECOMMENDATIONS

## 4.1 Discussion:

*Klebsiella pneumoniae (K. pneumoniae)* is one of the most common pathogens responsible for human infections. The emergence of highly antibiotic-resistant *K. pneumoniae* has become a major challenge facing clinical management and global public health, also can cause nosocomial infections. These isolates can carry virulence plasmids that harbor resistant genes (such as aminoglycoside resistant gene), according to the aminoglycoside resistance determinants in multi resistant *Escherichia coli* and *klebsiella.pneumonae* clinical isolated from (n=88) from Turkish and Syrian patients report at February 26, 2019. They found the *K. pneumoniae* the prevalence of resistance was 91.3% and 34.7% to gentamicin and amikacin, respectively. In *K. pneumoniae*, the incidence of resistance to tobramycin was reduced in our study because there is different in sample size, area and time when their study was done. However, we used same AST method. [12]

During the period between (2021-2022) USA Study in Aminoglycoside-resistance andcarbapenem-resistant genes in *Klebsiella pneumoniae* (n=169) they found the result was The most prevalent aminoglycoside-resistance gene, aac6 70.25% and one of their variance aac6-Ib0 (20.89%), followed by aph (30)-I (17.72%), then aac(3)-II (6.33%), aac (6)-Ib-cr (4.43%) and ant (200)-I (3.80%). less than (2%) of isolates harbored aac (3)-IV, aph (30)-II or rmtF. [31].In our study (n=50) we found the resistant genes at percentages Ant (12%), aac3 (42%), aac6 (4%), arm A (42%) and aph (0\0%). The results were matching with us in founding all genes. the significant different between us was that they found aph as the major aminoglycoside resistant gene and we do not found it. In our study we found arm A as major resistant gene and they did not found it. [11]

In 2021 Iran study (n=84) was detected aac(6) (94%)and arm A (83%). we detect five aminoglycoside resistance genes and found a high prevalence of arm A (42%) but they dis-match with us in the prevalence of aac (6) (12%).

## 4.2 Conclusion:

Our study found four aminoglycoside resistance genes from five, where arm A (42%) and acc3 (42%) are the major resistance genes followed by ant (12%), followed by acc6 (4%) and aph not detected.

The association between age group in the <1 year (40.0%) \n tobramycin and acc6 gene and association between blood sample (40.0%) and acc6 gene.

Found association between tobramycin age group (>1 year)

Found association between acc6 resistance gene and age group and type of sample.

## 4.3 Recommendation:

1. Large sample size should be performed in other studies for more information about aminoglycosides resistance.
2. Further studies in different geographical locations in Sudan.
3. More hospitals should include in further studies. And antimicrobial treatment should be done on the basis of sensitivity test to reduce the development of resistance strains.
4. Sample should be tested to detect virulence factors.
5. Further study should be done by use sequencing and analyzed by use bioinformatics.

# Reference


1- Jasim, SaadeAbdalkareem. "*Klebsiella* spp." PATHOGENIC BACTERIA 6: 76.

2-Ridgway, G. L. "Practical Medical Microbiology." Journal of Clinical Pathology1989 42.12: 1315.

3-Carroll, K. Janet ,B, and Stephen,M. Jawetzmelnick and adelbergs medical microbiology 27 E. McGraw-Hill Education, 2015.

4- Samaranayake, L. Essential microbiology for dentistry-E-Book. Elsevier Health Sciences, 2018.

5-Botelho-Nevers, E., et al. "Chronic nasal infection caused by *Klebsiella rhinoscleromatis* or *Klebsiella ozaenae*: two forgotten infectious diseases." International journal of infectious diseases2007, 11.5: 423-429.

6- Gelareh ,N. Amir ,P. Taghi Naserpour ,F., Peyman ,H. Molecular epidemiology of aminoglycoside resistance in clinical isolates of *Klebsiella pneumoniae* collected from Qazvin and Tehran provinces, Iran. Meegid 2018, doi:10.1016/j.meegid.2018.06.030

7-Levinson, Warren, and Ernest Jawetz. Medical microbiology and immunology: examination and board review. Appleton & Lange, 1996.

8- Jeongjin ,K.Ara, J.Ekachai,C. and Juhee, A. Assessment of antibiotic resistance in *Klebsiella pneumoniae* exposed to sequential in vitro antibiotic treatments, Annals of Clinical Microbiology and Antimicrobials: Kim et al. Ann ClinMicrobiolAntimicrob ,2016. 15:60 DOI 10.1186/s12941-016-0173-x.

9- YoheiDoi, M. Jun-ichi,W.Yoshichika, A. Aminoglycoside Resistance The Emergence of Acquired 16S Ribosomal RNA Methyltransferases, 2016. 523–537: http://dx.doi.org/10.1016/j.idc.2016.02.011 id.theclinics.com

10- osman, s. martafernández,m. buketyayla and luismartínez,m. aminoglycoside resistance determinants in multiresistanteescherichia coli and *klebsiella pneumoniae* clinical isolates from turkish and syrian patients.- ActaMicrobiologica et ImmunologicaHungarica, 2019.66 (3), pp. 327–335 DOI: 10.1556/030.66.2019.005 .

11-. Avent, M. Rogers, A. Cheng,C.andPaterson, D. Current use of aminoglycosides: indications, pharmacokinetics and monitoring for toxicity, -Internal Medicine Journa TheAuthorsInternal Medicine Journal ,2011. Royal Australasian College of Physicians442.:**https://doi.org/10.1111/j.1445-5994.2011.02452.x.** 14-Hall, Gerri S. "Bailey & Scott's Diagnostic microbiology, 13th edne138-e139.

12- Wachino, J. Yohei ,D. and Yoshichika,A. "Aminoglycoside resistance: updates with a focus on acquired 16S ribosomal RNA methyltransferases." Infectious Disease Clinics, 2020.34.4 887-902.

13- Davies, J.and Gerard. D."Bacterial resistance to aminoglycoside antibiotics." Trends in microbiology, 1997.5.6: 234-240.

14- Al Shara, M. . Emerging antimicrobial resistance of *klebsiella pneumonia* strains isolated from pediatric patients in jordan. Iraqi J Med,2011.7(2), 29-32.

15- Elbadawi, H. S., Elhag, K. M., Mahgoub, E., Altayb, H. N., & Hamid, M. M. A. Antimicrobial resistance surveillance among gram-negative bacterial isolates from patients in hospitals in Khartoum State, Sudan, 2019. F1000Research, 8(156), 156.

16- Elbadawi, H. S., Elhag, K. M., Mahgoub, E., Altayb, H. N., & Hamid, M. M. A. Antimicrobial Resistance Surveillance Among Gram Negative Bacterial Isolates from Patients in Khartoum State Hospitals,2018. bioRxiv, 486274.

17- Ahmed, A. A., Ibrahim, T. E., Kwami, W. S., &Idrees, H. M. Detection of aminoglycosides resistance among strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from patients with urinary tract infection, 2021.

18- Ahmed, S. M. S., & Musa, H. A. A. Detection of multidrug resistant and extended spectrum p-lactamases producing Klebsiella spp in Khartoum state, Sudan, 2019.

19- Yang, Y. Liu, D. Yang, M. Wang, J. Shen, H. Zhou, X.& Ma, X. A high throughput multiplex PCR assay for simultaneous detection of seven aminoglycoside-resistance genes in Enterobacteriaceae, 2013.

20- Ghotaslou, R.YeganehSefidan, F. Akhi, M. T. Asgharzadeh, M.&MohammadzadehAsl, Y. Dissemination of Genes Encoding Aminoglycoside-Modifying Enzymes and armA Among Enterobacteriaceae Isolates in Northwest Iran,2017.

21- El-Badawy, M. F., Tawakol, W. M., El-Far, S. W., Maghrabi, I. A., Al-Ghamdi, S. A., Mansy, M. S, &Shohayeb, M. M. Molecular identification of aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance genes among *Klebsiella pneumoniae* clinical isolates recovered from Egyptian patients. International journal of microbiology, 2017.

22- Huang, Y., Rana, A. P., Wenzler, E., Ozer, E. A., Krapp, F., Bulitta, J. B., Hauser, A. R., &Bulman, Z. P. Aminoglycoside-resistance gene signatures are predictive of aminoglycoside MICs for carbapenem-resistant *Klebsiella pneumoniae*, 2022.

23- Adesoji, A. T., Olatoye, I. O., &Ogunjobi, A. A. Genotypic Characterization of Aminoglycoside Resistance Genes from Bacteria Isolates in Selected Municipal Drinking Water Distribution Sources in Southwestern Nigeria,2019.

24- Soleimani, N. "Frequency distribution of genes encoding aminoglycoside modifying enzymes in uropathogenic*E. coli* isolated from Iranian hospital." BMC research notes, 2014. 7.1 (1-5).

25- El-Badawy, M. "Molecular identification of aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance genes among *Klebsiella pneumoniae* clinical isolates recovered from Egyptian patients." International journal of microbiology,2017 .

26- Mirnejad, R. "Identification of aminoglycoside resistance genes by Triplex PCR in *Enterococcus* spp. isolated from ICUs, 2016." Infez Med 24.3.: 222-9.

27-Dashti AA, Jadaon MM, Abdulsamad AM, Dashti HM. Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. Kuwait Med J. 2009 Jun 1;41(2):117-22.

28-Xueya ,Z. Qiaoling, L., Hailong. L.Wangxiao,Z.Changrui, Q.Zhewei, S, Hongmao, L.Junwan, L. Xi, L.Kewei,L.Teng, X.Hailin, Z.Changchong, L. and Qiyu,B. High-Level Aminoglycoside Resistance in Human Clinical *Klebsiella pneumoniae* Complex Isolates and Characteristics of armA-Carrying IncHI5 Plasmids Original research published,7 April 2021.doi: 10.3389/fmicb.2021.636396.

29- Yanqin,H. Amisha,P. Eric ,W. , Egon, A.Fiorella, K.,Ju¨rgen, B., Alan ,R. and Zackery ,P.Aminoglycoside-resistance gene signatures are predictive of aminoglycoside MICs for carbapenem-resistant *Klebsiella pneumonia*. J AntimicrobChemother ,2022.https://doi.org/10.1093/jac/dkab381 77: 356–363.

30- Mitra ,A. Payam ,B. Islamic Azad University Shahr-e-Qods BranchVirulence Factors Antibiotic Resistance patterns,and Molecular Types of Clinical Isolates of*Klebsiella Pneumoniae*,March 26th 2021.DOI: https://doi.org/10.21203/rs.3.rs-331542/v1

## Appendix I

**Data check list**

**Molecular Detection of Aminoglycoside Resistance Gene arm A, ant, Acc3, Acc6 and Aph of *Klebsiella .pneumonia*e isolated from clinical specimensin Khartoum State-Sudan 2022.**

**Abugogo Gaw Kafi Wanis.**

**Alfatih Alamin Bashir Taha.**

**Almojtaba Alfatih Ahmed Ibrahim.**

**Suliman Gorashie Suliman Khalil.**

**Omer Hassan Omer Ahmed.**

**Mustafa Yousif Adam Abaker.**

**Supervised by: Dr. SahrSaadeldinHagMohamed**

| | |
|---|---|
| Patient ID | Hospital name |
| Patient age | Gender |
| Type of sample | Isolate |

Culture result……

Sensitivity result ……

## Appendix II

Color plates and instrument

Figure 1: prepration of media

Figure 2: autoclave


Figure 3: Incubator culture


Figure 4: Hot air oven

Figure 5:***K.pneumoniae*** colonial morphology large, mucoid, pink colony, high convex, lactose ferment, smooth.

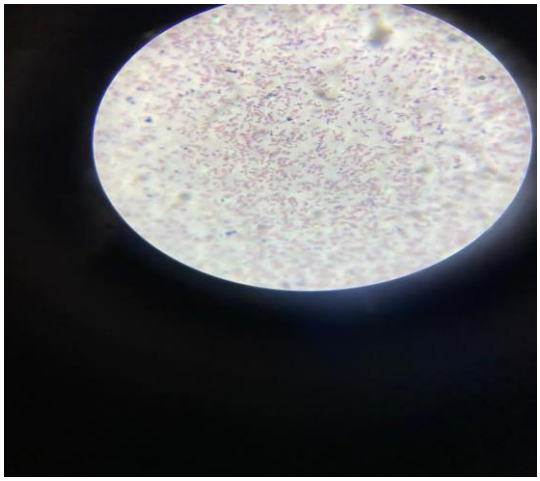

Figure 6: gram stain gram nagative short  bacilli

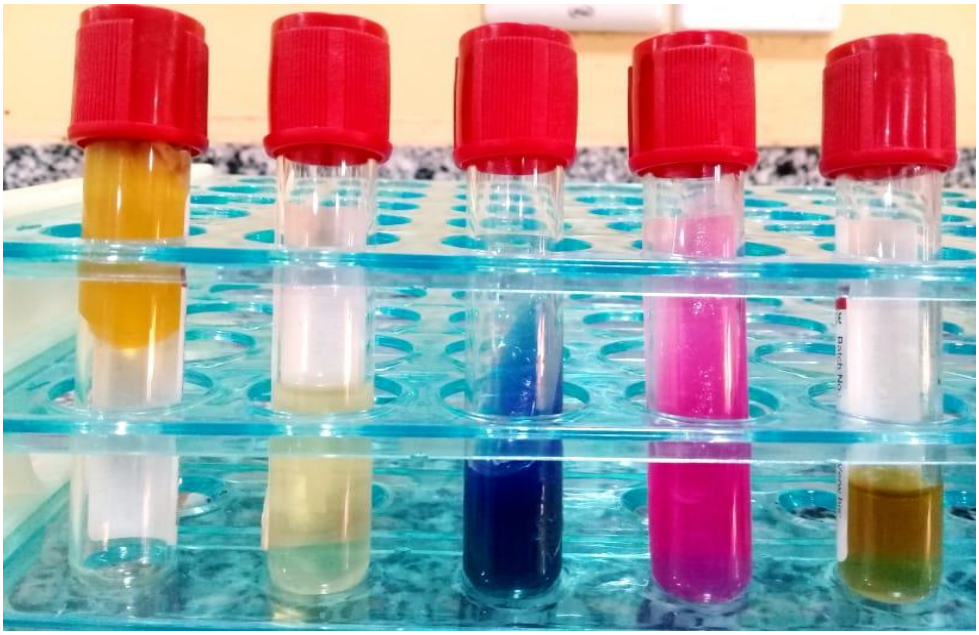

Figure 7: bio-chemical testresult of ***K.pneumoniae***[ KIA , Citrate , Urease , Indole , Motility]

Figure 8: Antimicrobials susceptibility using Kirby bauer disc diffusion method


Figure 9: Bio safety levels1 for add DNA to master mix and add primers


Figure 10: Bio safety level 2 to extraction of DNA

Figure 11: vortex mixer to mix sample

Figure 12: center fugue to separate sample

Figure 13: Thermal block incubator [Chem -Tech]


Figure 14: Thermo cycler PCR [Bio-Rad; C1000 touch, UK]

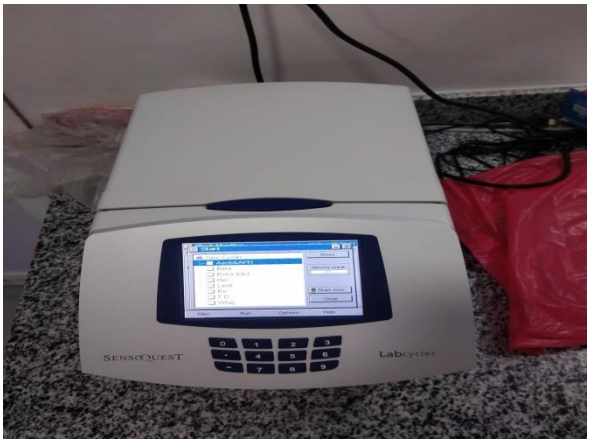


Figure 15: sensQuest [lab cycler]

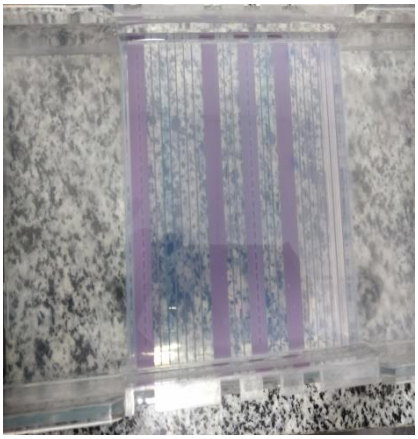

Figure16: loading of PCR products well of agarose gel using wet method

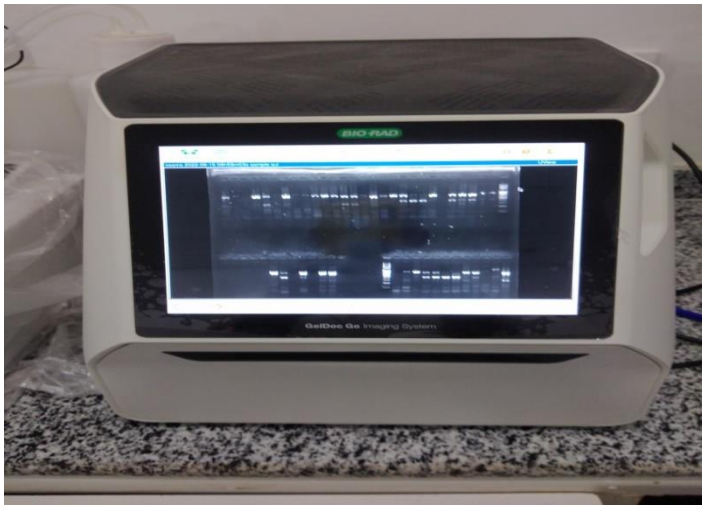


Figure17: Ultraviolet Trans- illuminator (Bio-Rad, UK)

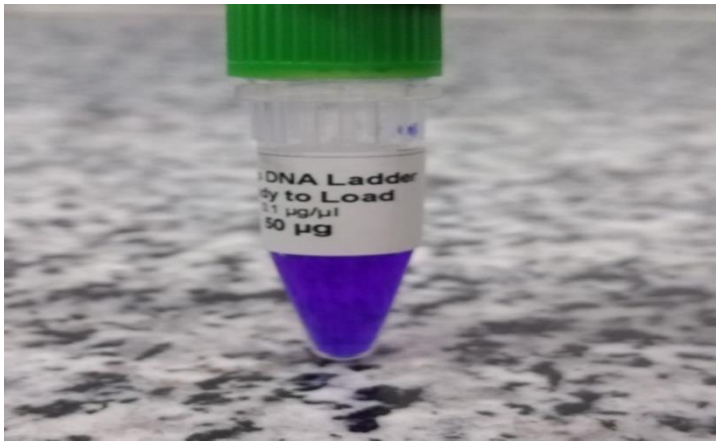


Figure18: DNA ladder

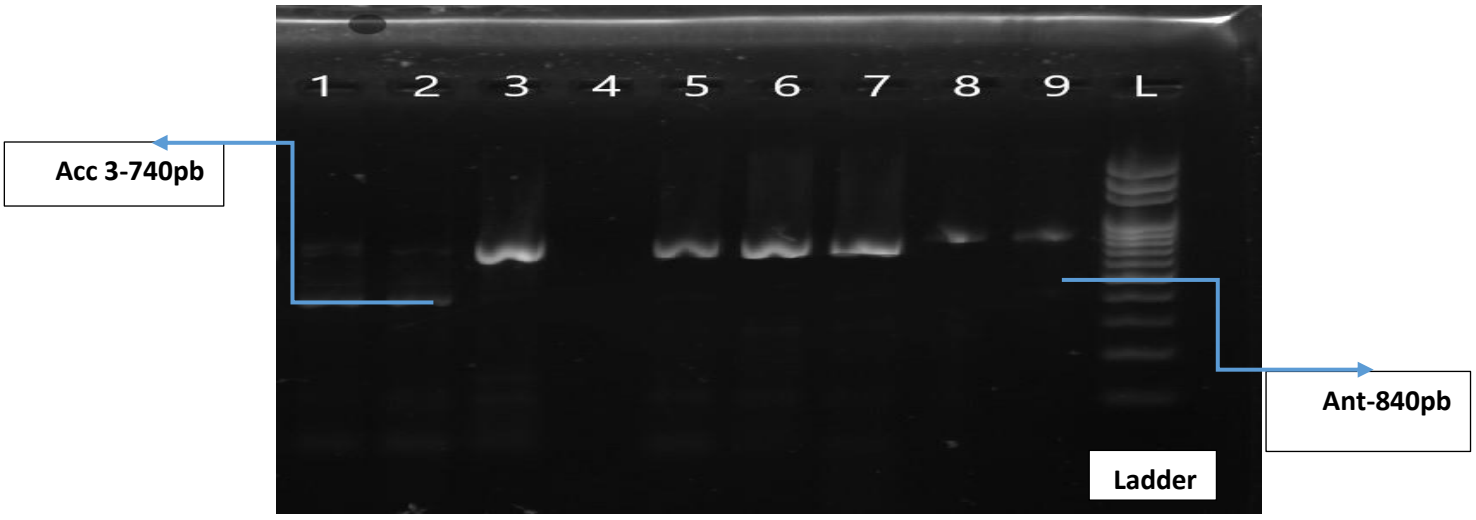


Figure19: product of PCR result

# APPENDIX III

**Table (3.1): Antimicrobial drugs, abbreviations, concentration, and zone size interpretation.**

| Name | Abbreviation | Concentration (mcg) | Interpretive Standards (mm) | | |
|---|---|---|---|---|---|
| | | | R (Resistant) | I (Intermediate) | S Sensitive |
| Amikacin | AK-30 | 30µg | ≤14 | 15-16 | ≥17 |
| Gentamicin | CN-10 | 10 µg | ≤12 | 13-14 | ≥15 |
| Tobramycin | Tob-10 | 10µg | ≤12 | 13-14 | ≥15 |

*** Performance standards for antimicrobial disk susceptibility, M100-S[32]**

# APPENDIX IV

## Preparation of reagents and culture media

Kliger Iron Agar (KIA):

PH (at 25°C) =7.4+/-0.2 Ingredients Gms/litter Peptic digest of animal tissue 15.0 Beef extract 3.00 Yeast extract 3.00 Proteose peptone 5.00 Lactose 10.00 Dextrose 1.00. Directions: Suspend 57.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. Mix and pour. Set as slope with butt.

Simmon's Citrate Agar:

pH (at 25°C) =6.8+/-0.2 Ingredients Gms/litter Magnesium sulphate 0.02, Ammonium dihydrogenphophate 1.00 Dipotassium phosphate 1.00 Sodium citrate 2.00 Sodium chloride 5.00 Bromothymol blue 0.08 Agar 15.00. Directions: Suspend 24.28 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 45°C- 50°C, mix well pour into sterile tubes

Christensen's Urea agar:

pH (at 25°C) =7.3+/-0.1 Ingredients Gms/litter Peptic digest of animal tissue 1.50 Dextrose 1.00 Sodium chloride 5.00 Monopotassium phosphate 2.00 Phenol red 0.012 Agar 15.00 Directions: Suspend 24.51 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50ml sterile 40% urea solution, mix well pour into sterile tubes. Indole broth: pH ( at 25°C) 7.5±0.2 Ingredients Gms / Litre Casein enzymichydrolysate 10.000 Sodium chloride 5.000

Directions: Suspend 15 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Semisolid media for motility: pH ( at 25°C) 7.2±0.2 Ingredients Gms / LitreTryptose 10.000 Sodium chloride 5.000 Agar 5.000 Final Directions: Suspend 20 grams in 1000 ml purified / distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow tubed medium to cool to 45-50°C in an upright position. Prepare Crystal Violet Stain: Dissolve 2 g crystal violet in 20 ml of 95% ethyl alcohol. Dissolve 0.8 g ammonium oxalate monohydrate in 80 ml deionized water. Mix the crystal violet and ammonium oxalate monohydrate solutions to make the crystal violet stain. Filter the stain if

necessary. prepare Safranin solution: Add 20mg safranin powder to a 100ml beaker. Pour 20ml distilled water in the beaker and make 0.1% safranin staining solution by constant stirring. Transfer 20mg of fast green dye in another 100ml beaker. ... Filter both the staining solutions to avoid particles. Acetone-alcohol decolorizer:

To make 1 litre, 500 ml of Acetone and 475 ml of Ethanol absolute add to 25 distilled water.

Mueller Hinton Agar:

pH (at 25°C) =7.3+/-0.1

Ingredients Gms/litter Beef, infusion from 300.00 casein acid hydrolysate 17.50 Starch 1.50 Agar 17.00 Directions: Suspend 38.0 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 45°C- 50°C, mix well pour into sterile petri plates. McFarland Standard Turbidity tube 0.5:

Ingredients Sulphuric acid 1 %, dehydrated barium chloride 1.16 g and distilled water 100 ml

Prepare 1% w/v solution of barium chloride by dissolve 1.16 g of dehydrated barium chloride in 100 ml of distilled water. Mix 19.9 ml of sulphuric acid with 0.1ml of barium chloride.

Preparation of 1X TAE buffer:

50X TAE (Thermo Scientific, Lithuania) was diluted to 1 L10X as a stock solution by adding 200 ml of 50X to 800 ml of distilled water, then 1L working solution of 1X was prepared using 100 ml of 10X diluted by 900 ml of distilled water.

Preparation of agarose gel:

2% agarose gel was prepared for each run by weight of 2 g of agarose powder (iNtRON Biotechnology, Korea) and add a small amount of 1X TAE buffer (Thermo Scientific, Lithuania) mixed and then complete to 100 ml, the mixture was heated by microwave for 1 min until a clear solution is produced, allowed to cool to 55ºC, then 2µl (0.2 for 10 µl) of Ethidium bromides (iNtRON Biotechnology, Korea) was added, mixed well and poured onto suitable gel tray that was equipped with combs to form wells for loading the PCR products. Any bubbles were removed and the gel was allowed to solidify at room temperature. After solidification, the comb was gently removed.