A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF ÇANKIRI KARATEKİN UNIVERSITY

MOLECULAR DETECTION OF URE A GENE IN HYPERMUCOVISCOUS MULTIDRUG RESISTANT KLEBSIELLA PNEUMONIAE ISOLATED FROM HOSPITALIZED PATIENTS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

BY

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ÇANKIRI

2022

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October 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

MOLECULAR DETECTION OF URE A GENE IN HYPERMUCOVISCOUS MULTIDRUG RESISTANT KLEBSIELLA PNEUMONIAE ISOLATED FROM HOSPITALIZED PATIENTS

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Master of Science in Biology

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October 2022

Hypermucoviscous *Klebsiella pneumoniae* is an emerging pathogen. In this study two hundred clinical samples were collected randomly from patients suffering from urinary tract infections. The collected samples were cultured on selective and differential media. The isolated bacteria were identified. Antibiotic susceptibility was detected by vitek system to detect the multidrug resistant bacterial isolates. String test was adopted to confirm the identification of hypermucoviscous type. Urease production by Hypermucoviscous *Klebsiella pneumoniae* was characterized phenotypically by Christensen medium. Ure A gene was detected using selective primers designated for this study. Polymerase chain reaction was adopted to detect the presence of urease coding gene. The resuls revealed that there were differences between the genetic and phenotypic methods to detect urease. PCR was more accurate as it detected the ure A gene in all the isolates (100%) while the phenotypic method results revealed that the positive urease occupied 70.5% of all the isolates.

2022, 38 pages

Keywords: Hypermucoviscous, MDR, PCR, *Klebsiella pneumoniae*, UreA

ÖZET

HASTANEDEKİ HASTALARDAN İZOLE HİPERMUKOVİSKUS ÇOKLU İLAÇ DİRENÇLİ KLEBSİELLA PNÖMONİDE URE A GENİNİN MOLEKÜLER TESPİTİ

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Ekim 2022

Hypermucoviscous Klebsiella pneumoniae ortaya çıkan bir patojendir. Bu çalışmada idrar yolu enfeksiyonlarından muzdarip hastalardan rastgele iki yüz klinik örnek toplanmıştır. Toplanan örnekler seçici ve diferansiyel besiyerlerinde kültüre edilmiştir. İzole edilen bakteriler belirlenmiştir. Çoklu ilaca dirençli bakteri izolatlarını tespit etmek için vitek sistemi tarafından antibiyotik duyarlılığı tespit edilmiştir. Hypermucoviscous Klebsiella pneumoniae tarafından üreaz üretimi, Christensen ortamı ile fenotipik olarak karakterize edilmiştir. Ure A geni, bu çalışma için belirlenmiş seçici primerler kullanılarak tespit edilmiştir. Üreaz kodlayan genin varlığını saptamak için polimeraz zincir reaksiyonu benimsenmiştir. Sonuçlar, üreazı saptamak için genetik ve fenotipik yöntemler arasında farklılıklar olduğunu ortaya koymuştur. PCR, tüm izolatlarda (%100) ure A genini tespit ettiği için daha doğruymuş, fenotipik yöntem sonuçları ise pozitif üreazın tüm izolatların %70,5'ini işgal ettiğini göstermiştir.

2022, 38 sayfa

Anahtar Kelimeler: Hipermukoviskus, MDR, PCR, *Klebsiella pneumoniae*, Üre

PREFACE AND ACKNOWLEDGEMENTS

First of all, we thank God for the most mercy for enabling us to present this project in the best form that we wanted to. I would like to extend my thanks, respect, and appreciation to my thesis advisor, Asst. Prof. Dr. Yaşar Kemal YAZGAN a lot for his help, patience, and taking care of us, and for making everything simple. He was always inspiring and encouraging us to move. I would like to extend my thanks, respect, and appreciation to my co-advisor, Asst. Prof. Dr. Marwa Hameed ALKHAFAJI for his invaluable supervision, support, and tutelage during the course of my degree.

Jaafar Jameel Kadhim AL-MAMOORI

Çankırı-2022

CONTENTS

LIST OF SYMBOLS

µg	Microgram
µL	Microliter
Cm	Centimeter
g	Gram
L	Liter
mg	Milligram
mL	Milliliter
Ng	Nano-Gram
Nm	Nanometer
^{o}C	Degree celsiu
Tm	Melting temperature

LIST OF ABBREVIATIONS

DNA	Deoxyribo nucleic acid
Hr	hour
MDR	Multiple drug resistance
PCR	Polymerase chain reaction
TBE	Tris, Borate, EDT
UTI	Urinary tract infection

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1. INTRODUCTION

Klebsiella pneumoniae is the causative agent of a lot of infectious diseases, such as urinary tract infections, bacteremia, liver abscesses, and pneumonias. It used to be that only people with compromised immune systems were at risk for developing a severe infection from *K. pneumoniae*, but the recent emergence and spread of hypermucoviscous strains has made healthy, immunocompetent people vulnerable to infection as well. As antibiotic resistance among *K. pneumoniae* strains has risen, it has become increasingly difficult to treat infections caused by these strains (Paczosa and Mecsas 2016). The hypercapsule, or mucoviscous exopolysaccharide bacterial coating, produced by hypermucoviscous *K. pneumoniae* strains is also known as a hypercapsule and is characterized by its increased strength in comparison to the usual capsule. The pathogenicity of hypervirulent *K. pneumoniae* may be considerably aided by this hypercapsule (Yeh *et al*. 2007)

Klebsiella pneumoniae is becoming increasingly recognized as a pathogen due to an increase in severe infections and a lack of effective treatments. These are roughly An issue has arisen due to the dissemination of *K. pneumoniae* strains that have acquired novel genetic features and become hypervirulent (HV) or resistant to antibiotics. Kaposi sarcoma was first discovered in the late 19th century and was at first called the bacteria Friedlander's sarcoma (Friedländer 1882). The cell is encapsulated, Gram-negative, and environment-dwelling, non-moving bacteria, such as on medical equipment, in surface waterways, and in soil (Rock 2014). It's crucial to note that *K. pneumoniae* easily colonizes human mucosa.surfaces, including the oropharynx and digestive tract, where its colonization seems to have had positive benefits (Bagley 1985, Dao 2014).

The ureolysis enzyme urease is crucial for many bacteria's ability to successfully infect humans. 8 In addition to serving as a source of nitrogen for bacteria and acting as an acid neutralizer to prevent acid stress damage, ureolysis produces ammonium (Scott *et al*. 2002).

The ureDABCEFG operon encodes the structural subunit ureABC and the accessory proteins ureDEFG that are required for urease activity by integrating nickel ions (Konieczna *et al.* 2013). Multiple bacteria, including Brucella suis and Helicobacter felis, possess duplicate copies of the urease operon. While ure-2 improves B. suis's ability to withstand acid, ure-1 is required for both in vivo persistence and acid resistance in the animal host (Bandarda *et al*. 2007)

Deleting ureB1 in H. felis completely eliminates urease activity, while inactivating ureB2 only reduces it (Pot *et al*. 2007). In contrast to *K. pneumoniae* MGH7857817 and NTUH-K2044, CG43 possesses two urease operons: ure- DABCEFG and ureABCEFGD, also known as ure-1 and ure-2, respectively (Wu *et al.* 2009).

This study aimed to detect the prevalence of ure A gene among hypermucoviscous Multi Drug Resistant Klebsiella pneumoniae isolated from hospitalized patients.

2. LITERATURE REVIEW

2.1 Enterobacteriaceae

Enterobacteriaceae are a diverse group of gammaproteobacteria that are either non-motile or move by means of peritrichous flagella. They are straight rod-shaped and spore-free, and they are facultative anaerobes, oxidase-negative, catalase-positive, nitrate-to-nitrite reducers, glucose-fermentors, and they have simple nutritional needs. They typically have rounded ends, dimensions of 2-4 m by 0.4–0.6 m, and a generation time of about 20–30 min (Strockbine *et al.* 2015). The distinction between members of this family has historically been made using biochemical traits, miniature experiments using various carbon and nitrogen sources, and, more recently, study of the 16S RNAr gene sequence. Therefore, in some circumstances, it may be required to apply further detection methods due to the limited selective power of 16S RNAr sequence analysis (Naum *et al.* 2008). The biggest shift in its taxonomic classification occurred in 2016, when Adeolu *et al*. (2016), based on phylogenetic analyses and preserved molecular characteristics analysis, recommended that the Enterobacteriales order, which had previously had a standard Enterobacteriaceae family, be renamed Enterobacterales and divided into seven families: Enterobacteriaceae, Yersiniaceae, Morganellaceae, Erwiniaceae, Pectobacteriaceae, Budviciaceae and Hafniaceae. Klebsiella remained in the enterobacteriaceae family (Morales *et al.* 2019).

2.2 Hypervirulent *K. pneumoniae*

According to differences in virulence, *K. pneumoniae* has recently been divided into classic *K. pneumoniae* (cKp) and hypervirulent K. pneumoniae (HvKp). Differentiating HvKp by its capacity to produce afterwards metastatic disease from cKp spread or to be present at multiple infection locations (Marr and Russo 2019). Liver abscess, Endophthalmitis, and meningitis are only few of the metastatic cancer complications that have been effectively managed with medical treatment (Siu *et al.* 2012 , Paczosa and Mecsas 2016, Gu *et al.* 2018). cKp is typically discovered and HvKp was initially described in nosocomial isolates, however individuals with community-acquired

illnesses are characterized hepatic abscesses from Singapore, Hong Kong, and Taiwan South Korea .Thus, the abbreviation HvKp was employed for nosocomial isolates that shared a MLST 11: multi-locus sequencing type (Siu *et al.* 2011, Lee *et al.* 2017).

This particular strain of HvKp infection has unique clinical traits. Most cases of this kind of HvKp are caused by illnesses picked up in the community, particularly pneumonia and liver abscesses. The only human risk factor for patients with this kind of HvKp infection who have liver abscesses is diabetes. The serotype predominance is distinct, despite the fact that several virulence factors from isolates of liver abscesses are the same as those from MDR-HvKp (Shon *et al.* 2013).

2.3 Virulence Factors

Well-known virulence factors for pathogenic *K. pneumoniae* include the capsule, lipopolysaccharide (LPS), fimbriae (types 1 and 3), and siderophores. The bacteria are enclosed in a capsule, which is an extracellular polysaccharide matrix. *K. pneumoniae* can generate a capsule with any of the serotypes K1 to K78, however K1 and K2 are associated with increased pathogenicity, hence they are of particular interest. HV strains are classified into two serotypes, with serotype K1 being the more common of the two and serotype K2 being the rarer. Hypercapsules are produced by HV strains, which increase the production of capsular material and result in a significantly larger capsule. LPS, an integral part of the outer leaflet of the outer membrane, can be produced by classical and HV *K. pneumoniae* strains alike, and can range from O-antigen serotypes 1 to 9 depending on the strain. (O1 to -9).Table 2.1, elucidate the mechanisms by which hypervirulent *K. pneumoniae* causes disease (Zhu *et al*. 2021).

Table 2.1 Virulence factors of hypervirulent *K. pneumoniae* (Zhu *et al*. 2021)

Category	Substance	Function
Iron acquisition	Siderophores: yersiniabactin, Enterobactin, Aerobactin ,salmochelin	Bind to environmental Fe3C iron
	Fur	Represses siderophores synthesis
	ABC-transporter: Kfu	Transport Fe3C into cytoplasm
Capsule	Wzi, Wza, Wzb, Wzc, Wzy, WbaP	Synthesize capsular polysaccharides
OMPs	PalA, LppA, OmpK35, OmpK36, KpnO	Protect against neutrophil phagocytosis
Adhesion	Type 3 fimbriae	Important for biofilm formation on abiotic surface
T6SS	PLD1, Tle1	Inject effector proteins into target cells,causing destruction
LPS	O-antigen	Avoid complement-mediated killing

Both forms of *K. pneumoniae* produce iron-scavenging siderophores as well as membrane-bound sticky structures known as type 1 and type 3 fimbriae. Of the siderophores, yersiniabactin is produced by roughly half of classical and nearly all HV strains, while enterobactin is produced by almost all strains. Aerobactin is the most highly expressed siderophore, whereas salmochelin is the least frequently generated by classical strains; instead, it is typically released by HV strains (Figure 2.1) (Paczosa and Mecsas 2016).

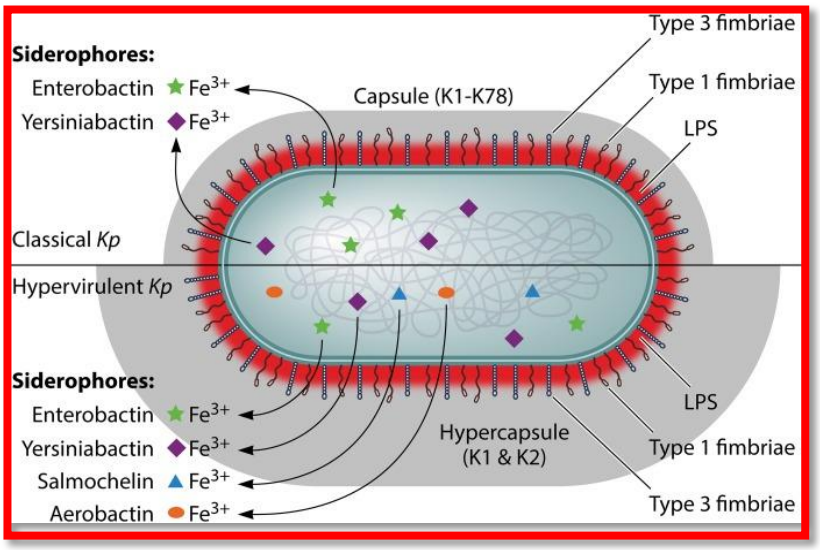

Figure 2.1 Classical and hypervirulent *K. pneumoniae* (Kp) strains share four well-characterized virulence factors (Paczosa and Mecsas 2016)

2.4 Urease

The ureolysis enzyme urease is crucial for many bacteria's ability to successfully infect humans. (Nielubowicz and Mobley 2010). The ammonium produced by ureolysis serves as both an acid neutralizer and a nitrogen supply for bacteria, preventing damage from acid stress (Scott *et al.* 2002). Helicobacter pylori urease may survive an excessively acidic pH in the stomach (Graham and Miftahussurur 2018). Yersinia enterocolitica (Bhagat and Virdi 2009) and *K. pneumoniae* (Maroncle *et al.* 2006) cannot colonize the mouse gastrointestinal system without urease activity. Urease activity aids in the production of urinary stones in the urinary tract where there is an abundance of urea, making a space for Proteus mirabilis to colonize (Schaffer *et al.* 2016).

The ureDABCEFG operon encodes the structural subunit ureABC and the accessory proteins ureDEFG that are required for urease activity by integrating nickel ions (Konieczna *et al.* 2013). The urease operon is carried by several bacteria, Brucella suis, Helicobacter felis, and others, in duplicates.

Ure-1 is required for both in vivo persistence in the animal host and acid resistance, in contrast to ure-2, which increases acid resistance in B. suis. Deleting ureB1 in H. felis completely eliminates urease activity, while inactivating ureB2 only reduces it (Bandara *et al.* 2007). In contrast to *K. pneumoniae* MGH7857817 and NTUH-K204418, CG43 possesses two urease operons, ure- DABCEFG and ureABCEFGD, which are ure-1 and ure-2, respectively (Pot *et al.* 2007) .

2.5 Urease Role in (UTI)

Both Proteus and Klebsiella types of bacteria, which can cause urinary tract infections, use a similar urease-dependent method. Infection caused by urease-positive bacteria may result in the formation of infection stones, which serve to encase and protect the pathogen. As a result of ammonium binding to magnesium ions and bicarbonate binding to calcium ions, the minerals struvite and carbonate apatite, respectively, precipitate to

form stones (Hedelin *et al.* 1985). Damage to the urothelium's protective glycosaminoglycan surface from ammonia is a contributing factor in bacterial urothelial infections (Musher *et al.* 1975). Therefore, some bacterial infections are promoted by urease-mediated pH shifts and harm to host epithelial cells (Rutherford 2014).

2.6 Urease Related Stones

According to the degree of occupancy, staghorn stones, which are enormous branching stones that partially or completely occupy the renal pelvis and renal calyces‹ of the collecting system (Healy and Ogan 2007). However, the phrase, The stone configuration is described by "staghorn," it lacks specified volume requirements and details regarding composition of stone (Preminger *et al.* 2005). It was once universally acknowledged staghorn stones made up 10–20% of the total urine volume nonetheless, this number has been decreased to 4 % in developed countries as a result of quick and efficient treatment for kidney stones (Diri and Diri 2018). Staghorn stones typically occur separately and are less common in men than in women, despite the fact that kidney stones are more common in men (Johnson *et al.* 1979 , Niels *et al.* 2003).

The term "staghorn stone" historically refers to struvite stone because infecting stones account up 49–68% of staghorn stones (Resnick and Boyce 1980 , Viprakasit *et al.* 2011). Magnesium, ammonium, and phosphate make up struvite stone, which was first identified by the Swedish geologist Ulex in 1845. Struvite stone is intimately associated to urinary tract infections brought on by Proteus, Klebsiella, Pseudomonas, and Staphylococcus bacteria, which produce urease (Heimbach *et al.* 2002). Figure 2.2 clarifies the staghorn stone of an infected female.

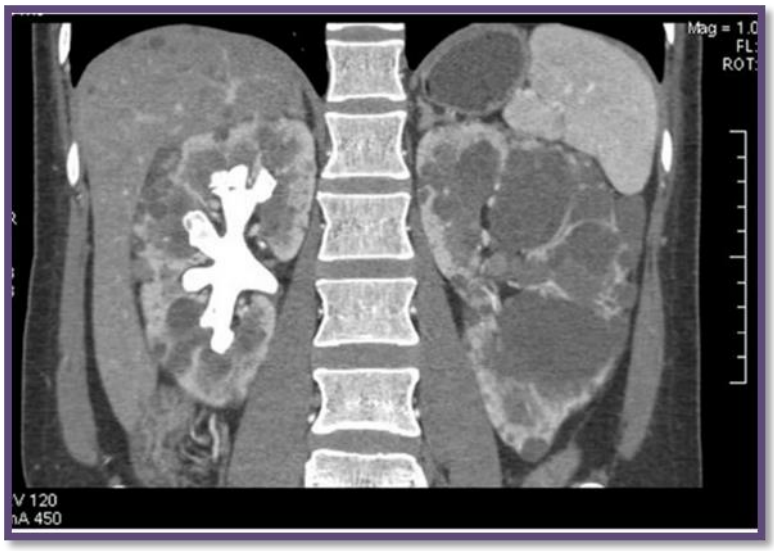

Figure 2.2 CT images of a complete staghorn calculus in the right kidney in an ADPKD female. (the staghorn calculus and polycystic kidney in the coronal plane) (Mao *et al.* 2013)

2.7 Urease Related Catheter Associated (UTI)

In developed countries, catheter-associated urinary tract infections (CAUTIs), which account for approximately 1 million cases yearly, are one of the most prevalent illnesses linked with healthcare. CAUTIs have a significant morbidity and mortality rate, and they are caused by a wide range of bacteria, making empiric treatment difficult. Catheter failure due to blockage is also more likely when uropathogens that generate urease cause either symptomatic catheter-associated urinary tract infections or asymptomatic catheter colonization. By hydrolyzing urea in urine into ammonia and carbon dioxide and causing crystals to develop on the catheter surface, the enzyme urease encourages catheter blockage (Duran Ramirez *et al.* 2022). Both symptomatic urinary tract infections (UTIs) and asymptomatic bacteriuria (ASB) are more common in patients with urinary catheters, and those who need to have their catheters in for extended periods are at the greatest risk (Nicolle 2019). ASB can also cause catheter blockages in people who have chronic indwelling urinary catheters, which reduces their quality of life by causing recurrent device failure and increasing the risk of further comorbidities like pyelonephritis and bacteremia (Stickler and Feneley 2010).

Encrustations, which form after infection with CAUTI pathogens like *Klebsiella pneumoniae*, *Proteus* species, and Providencia species, are a common cause of catheter blockages (Broomfield *et al.* 2009).

Urease specifically hydrolyzes urea, which is contained in urine, into ammonium and carbon dioxide, which significantly raises the pH of urine and causes the development of crystalline precipitate. Urinary catheter encrustations are caused by the buildup of crystalline precipitates on the catheter surface (Figure 2.3), and if the infection is not treated, it may cause catheter obstructions and device failure (Armbruster *et al.* 2017).

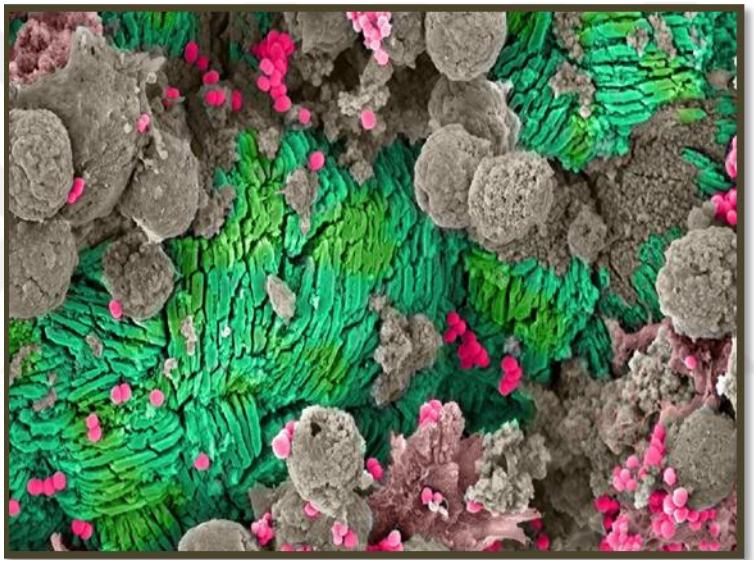

Figure 2.3 Catheter Encrustation (Duran Ramirez *et al.* 2022)

Asymptomatic catheter-associated bacteriuria due to Staphylococcus aureus, as seen using a scanning electron microscope (SEM) of crystalline precipitates creating an encrustation (green) on a catheter (pink). Host immune cells cluster around the encrustation and bacterium (gray) (Duran Ramirez *et al.* 2022).

3. MATERIALS AND METHODS

A total of two hundred clinical samples will be collected from hospitalized patients in Iraq during the period extended from March to June 2022. Clinical specimen of urine, wounds and catheter associated urine were collected from the Medical City of Alkadhymain in Baghdad.

3.1 Materials

3.1.1 Chemical and biological materials

The Chemical and biological materials that used in this study are mentioned in Table 3.1.

Table 3.1 Chemicals and biological materials

Chemical	Company / Origin
100 DNA Ladder	Gene Dires/ USA
Agarose	USA
Agar-Agar	Hi-Media / India
Absolute ethanol (99%)	Merk / England
AccuPower PCR PreMix	Bioneer / Korea
Deionized sterile Distillate water	Bioneer / Korea
Ethyl Alcohol (Ethanol)	GCC / England
Geneaid DNA Isolation Kit (Bacteria)	Geneaid/ Thailand
Glycerol	GCC / England
Gram stain reagents	USA
Hydrochloric acid (HCl)	BDH / England
Red Safe	USA
MacFarland standard	
Nuclease Free Water	Promega, USA
Normal Saline	S.D.I / Iraq
Sodium hydroxide (NaOH)	BDH / England
Tris-Borate-EDTA Buffer (TBE 40X)	Bio Basic INC / Canada
Urea	

3.1.2 Culture media

3.1.2.1 Ready to use culture media

The culture media used in this study are listed in Table 3.2.

Table 3.2 The culture media used in this study

Culture media	Company / Origin
Brain-Heart Infusion broth	Hi-media / India
Eosin methylene blue	Hi-media / India
MacConkey agar	Hi-media / India
Muller Hinton agar	Hi-media / India
Nutrient agar	Oxoid / England
Nutrient broth	Oxoid / England
Urea base agar	Hi-media / India

All media listed in table (3.3) were prepared according to the manufacturer instructions, pH was adjusted to 7 with 0.1N NaOH or 0.1N HCl, then sterilized by autoclaving at 121^{o}C/15 pounds/inche^{2} for 15 minutes, then the prepared media distributed into sterile tubes or Petri dishes.

3.1.2.2 Prepared culture media

3.1.2.2.1 Urease production

Urea agar medium (Collee *et al*. 1996). This medium consists of: 95 mL urea base agar (which was prepared according to the manufacturer's instructions; pH was adjusted to 6.8-6.9 and autoclaved) and 5 mL of 40% filtered urea solution was added to the cooled sterilized urea base.

3.1.3 Material used in agarose gel electrophoresis and in PCR amplification

3.1.3.1 (TBE) buffer

TBE buffer (10 X) was supplied by (Bio Basic INC / Canada), consisted of Tris base, boric acid and EDTA. The pH was adjusted to 8.3 then 100 mL of TBE (10 X) was added to 900 mL of D.W. to obtain 1X solution used for agarose gel electrophoresis.

3.1.3.2 DNA ladder (100 base pair)

100 bp DAN sample was obtained using the Presto Mini gDNA Bacteria Kit, and a DNA ladder was purchased to use in determining the size of double stranded DNA ranging from 100 to 1500 bp. The supplier, GeneDires, provided the DNA ladder as a liquid.

3.1.3.3 PCR Accupower Premix

The PCR premix that was supplied by the manufacturer (BIONEER) includes the following components: 1U of top DNA polymerase, deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), 1X reaction buffer containing 1.5 mM MgCl2, stabilizer, and tracking dye.

3.2 Methods

3.2.1 Bacterial isolation

Klebsiella was successfully isolated by culture from the collected clinical samples on selective and differential media. MacConkey agar was inoculated with 200 different clinical samples, and the plates were incubated at 37 degrees Celsius for 24 hours. After the colonies had grown, more tests were run on them.

3.2.2 Bacterial identification

Both microscopic and macroscopic characteristics on selective and differential media were used to determine the taxonomy of bacterial isolates, as stated by MacFaddin (2000). Vitek system confirmed the identification to the species level.

3.2.2.1 Cultural characteristics on selective and differential media

The organisms were cultured on MacConkey agar media and incubated overnight at 37°C. The pale colonies were neglected and the pink colonies were sub-cultured on Eosin methylene blue (EMB) agar to differentiate between *Escherichia coli* (green metallic sheen) and *K. pneumoniae* (purple colonies) after incubation at 37^{o}C. On MacConkey agar *Klebsiella* appeared as large convex mucoid pink colonies (Holt *et al.* 1994).

3.2.2.2 Microscopic examination

In order to examine the bacterial morphological characteristics and response to Gram stain, a part of the suspected colony from the positive culture was transferred and fixed on a clean slide, then Gram's staining was carried out. Thereafter, it was examined under a light microscope using oil immersion.

3.2.2.3 Identification of *Klebsiella* isolates by vitek system

Bacterial isolates were identified and tested for sensitivity using Vitek. The whole thing folds down into two small pieces. There are five individual components that make up the instrument and one computer:

1. Keypad.

2. In a period of 70 seconds, the sample was transferred from the tested tube to the kit via fill door.
3. Transfer tube was severed from the kit and loaded into the incubator a few minutes later through the load door.
4. Doors for Entry by Users (Here, we tallied up all the alterations brought on by bacterial expansion to arrive at our final conclusion).
5. Discarded Material Exit (Here, we're picking up the kit after it's been used).

3.2.2.4 Principle

The Vitek is an automated microbiology system utilizing growth-base technology, the system has colourimetric reagent cards that are incubated and interpreted automatically. Vitek compact is a fluorometric analysis. There is room for 64 individual test substrates on each of the reagent cards. Measuring metabolic processes on substrates includes acidification, alkalinization, enzyme hydrolysis, and growth despite the presence of inhibitors.

Both sides of the card are coated with an optically clear layer that lets just the right amount of oxygen through while yet keeping the card airtight. That eliminates the risk of coming into contact with the organism-substrate combinations. Vaccines can be administered with the use of a transfer tube that is included with each card. Before or after inserting a sample card into the system, the bar code can be scanned to obtain information about the product kind, lot number, and expiration date.

3.2.2.5 Urease production test

Christensen medium was used to detect the ability of bacterial isolates to produce urease enzyme. This test shows the bacterial ability to produce urease enzyme. The bacterial isolate was streaked on urea agar slant (3.1.3 II), and then incubated at 37^{o}C for 18-24 h. The positive result was evidenced by the change in the medium color from deep orange to pink.

3.2.3 Detection of hypermucoviscous *K.pneumoniae* by string test

The String Theory Exam After an overnight incubation at 37°C, a colony was "stretched" using a bacteriology inoculation loop. Positive results on the string test indicated the presence of a mucoid string longer than 5 mm (Li *et al*. 2014).

3.2.4 Bacterial preservation

3.2.4.1 Short term storage

Single pure colony of each bacterial isolate was streaked on the nutrient agar culture plates and on the nutrient agar slants, incubated at 37^{o}C for 24 hr. and were stored in the refrigerator at 4^{o}C, for one to three months for plate and slants respectively (Prescott and Harley 2002).

3.2.4.2 Long term storage

1. **Stab culture method:** A pure isolated colony (18hr) was stabbed into nutrient agar, then incubated at 37^{o}C for 24hr and stored in dark place at room temperature (Vandepitte *et al.* 2003).
2. **Glycerol method:** A nutrient broth was inoculated by loopful of overnight pure bacterial culture, and incubated at 37^{o}C, after 18hr glycerol was added to the inoculum in a final concentration of 15-30% and stored at(-20^{o}C) for 12-18 months.

3.2.5 Genetic study

3.2.5.1 DNA extraction

DNA extracted using Genomic DNA mini kit, (Geneaid, Thailand) protocol for gram negative bacteria was used as the following:

1. Bacterial broth was diluted by adding 1 mL to a 1.5 mL micro centrifuge tube and spinning it at 16,000 rpm for 1 minute. The supernatant was thrown away. After adding 180 µL of GT Buffer, the cell pellet was resuspended using a vortex mixer. Followed by the addition of 20 µL of Proteinase K Then Incubated at 60ºC for 15 minutes.
2. GB buffer (200µL) was added by shaking vigorously for 5sec then incubated at 70˚C for 10min. during incubation, the tubes were inverted every 3min.
3. Absolute ethanol (200µL) was added and mixed by shaking vigorously, then transferred to a mini column and centrifuged at 13000 rpm for 2min.
4. The collection tube was discarded and placed the GD column in a new one.
5. W1 buffer (400µL) was added then centrifuged at 13000 rpm for 30 secs and the flow-through was discarded.
6. Washing buffer (600µL) were added, centrifuged at 13000 rpm for 30 secs and the flow-through was discarded.
7. To dry the column matrix, the GD column centrifuged at 13000 rpm for 3min.
8. Elution buffer (100µL), previously incubated at 70˚C for 10min, was added to GD column then transferred to a clean microfuge tube and let stand for 3-5 min. then centrifuged at 13000 rpm for 30 sec.
9. The DNA was stored in a deep freezer until PCR analysis is carried out.

3.2.5.2 Quantitation of DNA by qubit 4.0

The assay is specific for starting sample concentrations between 10 pg/ µL and 100 ng/ µL, and it favors double-stranded DNA (dsDNA) over RNA. The test can be run in a room temperature environment, and the signal remains constant for three hours. The assay is very forgiving of common impurities such salts, free nucleotides, solvents, detergents, and protein. The standard and short procedure showing in Figure 3.1.

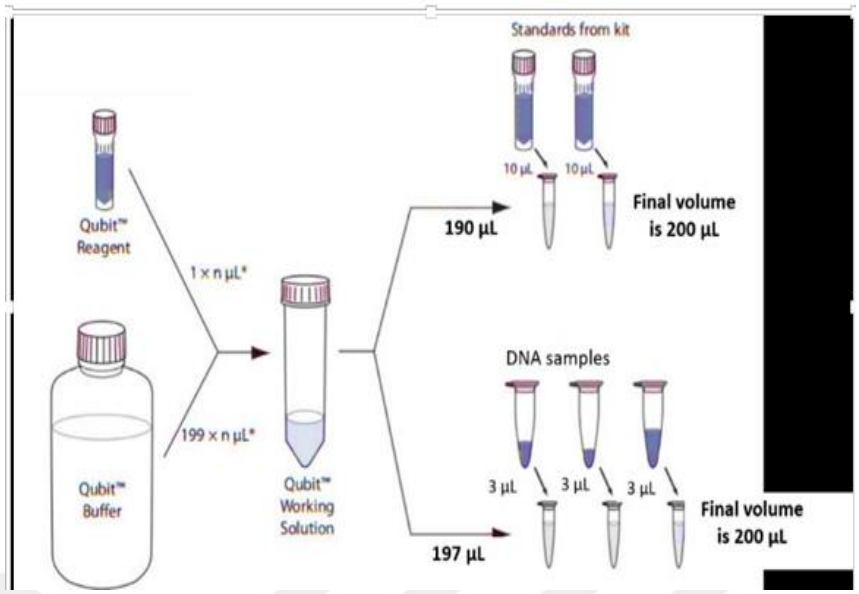

Figure 3.1 Procedure of DNA quantitation by Qubit 4.0

1. The Qubit® dsDNA HS Reagent was diluted in Qubit® dsDNA HS buffer at a 1:200 ratio to make the Qubit® working solution.
2. Each standard-designated tube has had 190 µL of the Qubit® working solution added to it, and then 10 µL of each of the specified standard solutions has been added and vortexed into the tubes.
3. Each sample tube has 197 µL of Qubit® working solution added to it, and then 3 µL of sample is added..
4. After 3 minutes of incubation at room temperature with vigorous vortexing, the entire composition was checked.
5. The Qubit instrument has been outfitted with standards tubes for the purpose of drawing concentration curves.
6. One by one, sample tubes have been added so that their dsDNA concentrations can be read.

3.2.5.3 PCR Amplification of biofilm coding (*UreA*) gene

The primers used to detect urease gene (*ureA*) in hypermucoviscous *K. pneumoniae* were designated in this study, PCR technique was adopted to investigate the presence of this gene in the isolated clinical *K.pneumoniae* (Table 3.3).

Table 3.3 Designated Primers and their sequences used for amplification of *ureA* gene

Primer Name	Sequences (´5 ⟶ ´3)	Tm (°C)	Size (bp)
ureA- F	CGAGAAAAAGACAAGCTGTTG	61.6	297 bp
ureA- R	GGCGCTACCTTAGATAATCG	60.9	

These primers were provided in a lyophilized form. They were dissolved in sterile deionized distilled water to give a final concentration of100 pmol/µL. Primer stock and working portion were stored in the deep freezer until used in PCR amplification.

The extracted DNA, primers and PCR premix, were thawed at 4^{o}C, vortex and centrifuged briefly to bring the contents to the bottom of the tubes. There was 12.5 µL of PCR premix, 1.5 µL of each primer, and 4 µL of template DNA utilized to make a total volume of 25 µL for the PCR reaction.

De-ionized distilled water was used to fill up the remaining space, and the mixture was then vortexed to kill any bacteria. The template DNA was left out of the negative control, and it was replaced with distilled water. After placing the PCR reaction tubes into a thermo-cycler PCR instrument, the contents were briefly centrifuged to mix and bring the contents to the bottom of the tubes as indicated in Table 3.4.

Table 3.4 Program used to amplify the *ureA* gene

Stage	Temperature ^{o}C	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	35
Annealing	50	45 sec	
Extension	72	30 sec	
Final extension	72	7 min	1

3.2.5.4 Determination of PCR product specificity

Electrophoresis on an agarose gel was used to determine whether or not PCR results were reliable. For the PCR specificity assay, a 1.5% agarose gel was produced by dissolving 1.5 g of agarose powder in 100 mL of 1X TBE buffer and boiling the

mixture. Once cooled to 50-60oC, 5 µL of safe red dye was added and mixed well, and the gel was placed into a jar and chilled to 20°C. The comb was used to make multiple wells in the agarose gel at one end, around 5-10 mm from the end, and then removed once the gel had completely hardened.

The bottle was transferred to the electrophoresis apparatus. After loading the agarose electrophoresis gel with 6 µL of the 100 bp DNA ladder in either the top left or bottom middle well, we added 10 µL of each PCR product to the remaining wells. Thereafter, the customized lid was placed back on the electrophoresis tank, and the electric current was matched (70 volt for 1.5 h). The red safe stained bands in gel were visualized using Gel documentation system (Maniatis *et al*. 1982).

4. RESULTS AND DISCUSSION

4.1 Hypermucoviscous *Klebsiella pneumoniae* Isolation and Identification

Hypermucoviscous *Klebsiella pneumoniae* isolates were isolated from two hundred urine samples. Clinical samples which were collected from patients and cultured on selective and differential media. *Klebsiella* was isolated from other bacterial cultures by using MacConkey agar to help distinguish between them. Macroscopic inspection of MacConkey agar after 18 hours of incubation at 37oC revealed two types of colonies: pink colonies from lactose fermenters and pale colonies from non-lactose fermenters. From a total of 119 bacterial isolates obtained from urine samples, 78 (65.54%) were lactose fermenters and 41 (34.45%) were lactose non-fermenters (Figure 4.1).

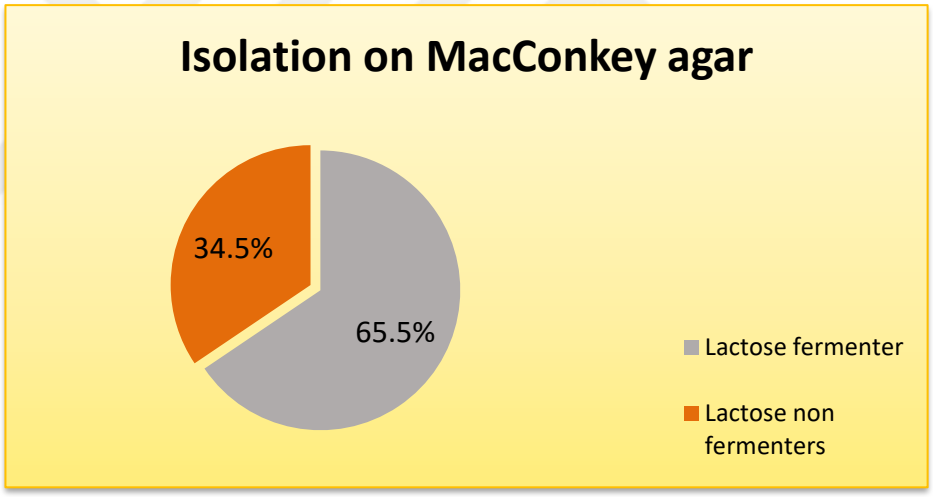

Figure 4.1 Percentages of lactose fermenting bacteria and non-lactose fermenting bacteria isolated from clinical samples

In addition to macroscopic characteristic on these media, microscopic examination used to identify the Gram stainability, shape and arrangement.

The pink colonies were cultured on chromo agar for further identification; *Klebsiella* appeared as large mucoid convex pink colonies on MacConkey agar. Out of 78 lactose fermenters; Thirty eight *Klebsiella* isolates were identified in this study (Figure 4.2).

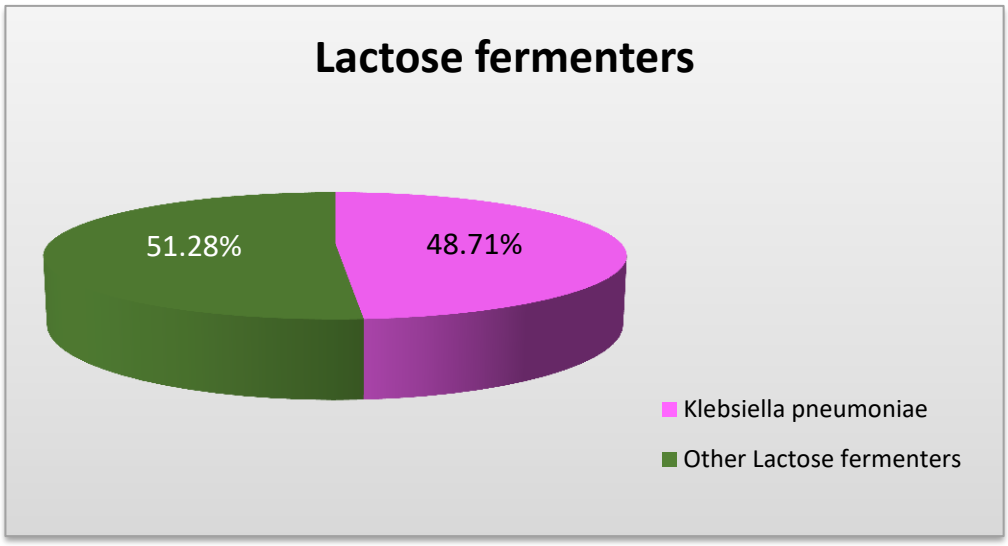

Figure 4.2 Percentage of Klebsiella isolates among lactose fermenters isolated from urine samples

Klebsiella pneumoniae can be identified by its ability to ferment lactose on MacConkey agar and by the viscosity of its colonies; encapsulated strains of *Klebsiella* spp. are likewise mucoid in appearance (Figure 4.3).

Figure 4.3 Large pink convex mucoid colonies of *Klebsiella spp* on MacConkey agar after incubation at 37°C for 24 hr

In order to confirm the identification to species level; Vitek system was used. The result appeared that the isolated bacterial species in this study was *Klebsiella pneumoniae.* Vitek system was used to detect the multidrug resistant (MDR) isolates and the results revealed that out of 38 *K. pneumoniae* isolates; 20 isolates (52.63%) were MDR; that

were resistant to at least three or more classes of antibiotics. Numerous illnesses, including pneumonia, bloodstream infections, and urinary tract infections, can be brought on by Klebsiella pneumoniae.

Bacterial culture is the gold standard to confirm the causative agent of a particular infection. So when the clinical samples of urinary tract infections were cultured on selective and differential media; the resulted bacterial isolates gave a complete picture about the causative agents of this type of infections. It is not astonishing to isolate *K. pneumoniae* from UTI hospitalized patients Community-acquired *K. pneumoniae* infections are public.

K. pneumoniae has the capability of resistance genes acquisition and to convert gradually more challenging to be treated. Inaccurate diagnosis and ineffective treatments could be one of the causes of a potential treatment failure. It is crucial to include differential diagnosis in laboratory diagnostic methods in order to establish the etiology since similar symptoms can result in regular therapies based on syndromic techniques, which are frequently used in underdeveloped countries. Treatment should ideally be based on the findings of the bacteriological examination as the doctor is rarely able to make an etiological diagnosis on the basis of clinical grounds alone. In this instance, the possibility of *K. pneumoniae* infection of the respiratory tract could confound bacteriological diagnosis (Sasaki *et al.* 2017, Rakotondrasoa *et al.* 2022).

4.2 String Test Results

Hypermucoviscous *K. pneumoniae* phenotype was detected using string test. It has been confirmed the positive results by the formation of continuous mucoid thread between the colony and the loop greater than 5 millimeter (Figure 4.4).

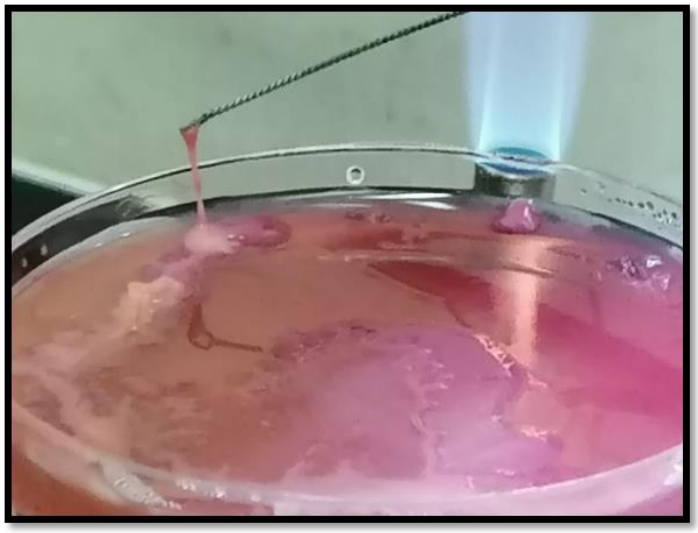

Figure 4.4 Hypermucoviscous *K. pneumoniae* phenotype detection by string test for isolate K9 on MacConkey agar: a positive result appeared as a viscous string larger than 10 mm in length

String test results revealed that out of 38 *K. pneumoniae* isolates 17 (44.73%) of them were positive for string test; this finding was in accordance to that obtained by Cheng *et al.* (2018), were the string positive isolates accomplished 36.36% of the isolated bacteria.

Out of 38 *Klebsiella pneumoniae*; 17 isolates were hypermucoviscous and 21 were classic *K. pneumoniae* (Figure 4.5).

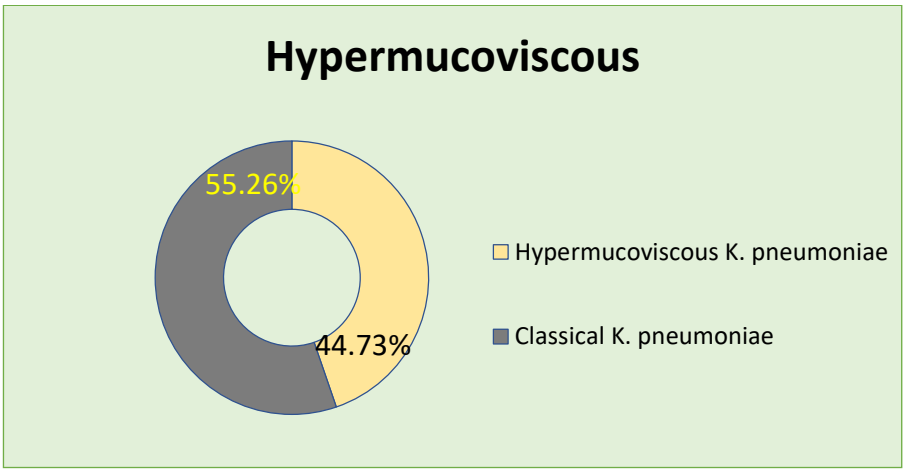

Figure 4.5 Percentage of Hypermucoviscous and classical *K. pneumoniae*

When classical *K. pneumoniae* strains infect immunocompromised patients, such as those suffering from HIV/AIDS, they frequently result in serious illnesses including pneumonia, bacteremia, or meningitis.

from malignancies or diabetes (Meatherall *et al.* 2009). The posture and carriage and expression of drug resistance do not make *K. pneumoniae* more virulent. despite making them more challenging to treat, strains. However, *K. pneumoniae* strains that can cause since the 1980s severe infections have also occurred in otherwise healthy people. attracted the attention of people.

These varieties are regarded as hypermucoviscous in contrast to traditional *K. pneumoniae* strains because their capacity to infect both immune-suppressed people and healthy people communities, as well as the rising prevalence of these illnesses to be invasive, i.e., they can cause liver infection (Liu *et al.* 1991, Patel *et al.* 2014). This increased virulence is associated with the acquisition of a 200–220 kb plasmid encoding siderophores and genes to boost capsule formation.

4.3 Urease production by Hypermucoviscous *K.pneumoniae*

Urease enzyme production by hypermucoviscous *K. pneumoniae* was detected using Christensen medium. The results showed that out of 17 MDR hypermucoviscous *K. pneumoniae* isolates; twelve isolates were positive for urease enzyme production phenotypically (Figure 4.6).

Figure 4.6 Phenotypic Urease test

Christens medium cultured with two MDR hypermucoviscous *K. pneumoniae* isolates, the figure clarifies the positive result of the isolate 46 that produced urease enzyme (Pink) and the negative result of the isolate 40 (Yellow).

Urease enzyme split urea to ammonia thus raising pH resulting in the colour change of indicator from yellow to pink.

Not all bacterial genera that can produce urease enzyme can splits urea. While all Proteus species, Providencia species, and Morgenalla species generate the urea-splitting enzyme not all Staphylococcus and Klebsiella spp.produce urease (Flannigan *et al.* 2014, Torricelli and Monga 2020).

One of the earliest techniques involved cultivating microorganisms on urea-containing medium (Christensen's urea media) in order to determine bacterial ureolytic activity. For species like Proteus sp., this is the most widely used qualitative technique. Results may be obtained for this particular bacterial species even 4 hours later. a reworking of Christensen method enables speeding up assays (Christensen 1946).

4.4 Results of the Genetic Study

4.4.1 DNA extraction from *Klebsiella* isolates

Genomic DNA extraction kit was used to extract DNA from MDR hypermucoviscous K. pneumoniae isolates, and the qubit 4.0 assay was used to characterize the extracted DNA samples to ensure their purity and quality. The results showed that all of the extracted DNA samples were pure, with concentrations greater than 452 ng/ μL

4.4.2 Polymerase chain reaction (PCR) technique for *ure A* gene detection

Multi drug resistant hypermucoviscous *K. pneumoniae* isolates were detected for the presence of ure A gene (*ure A*), using PCR technique, and the results revealed that all clinical MDR hypermucoviscous *K. pneumoniae* isolates (100%) carried *ure A* gene, that they amplified *ure A* gene and the PCR product of 297bp was detected using agarose gel electrophorsis and photographed using ultraviolet (UV) transilluminator (Figure 4.7).

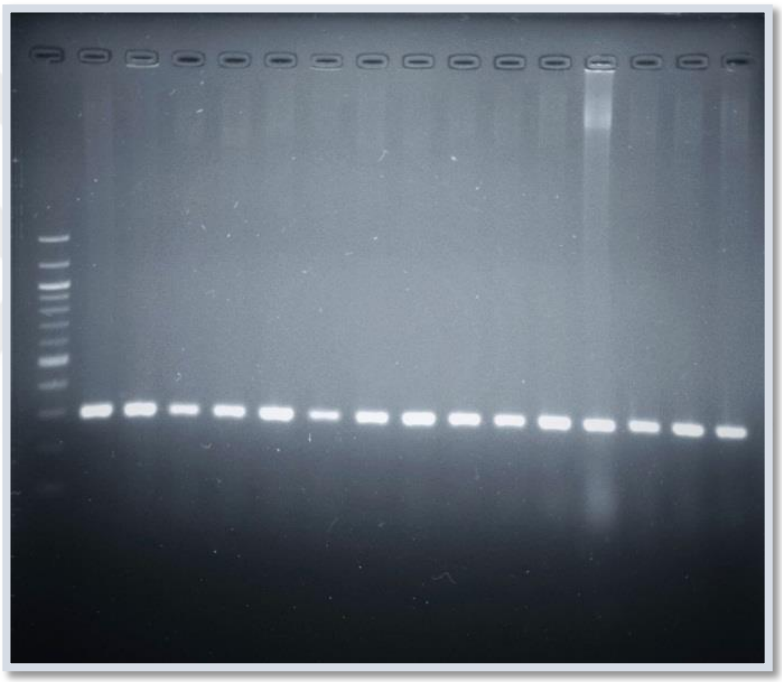

Figure 4.7 Agarose Gel Electrophorsis of amplified PCR product of *ure A* gene (297bp), of clinical MDR hypermucoviscous *K. pneumoniae*

Agarose (1.5%), TBE (1x), 5v/cm for 1h stained with RedSafe. M: The DNA molecular weight marker (100bp) ladder, 1-15 *ure A* positive PCR products of the isolates: K11, K12, K13, K24, K25, K27, K18, K29, K30, K33, K34, K35, K36, K42, and K46 respectively

The results of this study were illustrated in Figure 4.8 that clarified a comparison between phenotypic detection of urease enzyme production and genetic detection of the gene responsible for the urease coding.

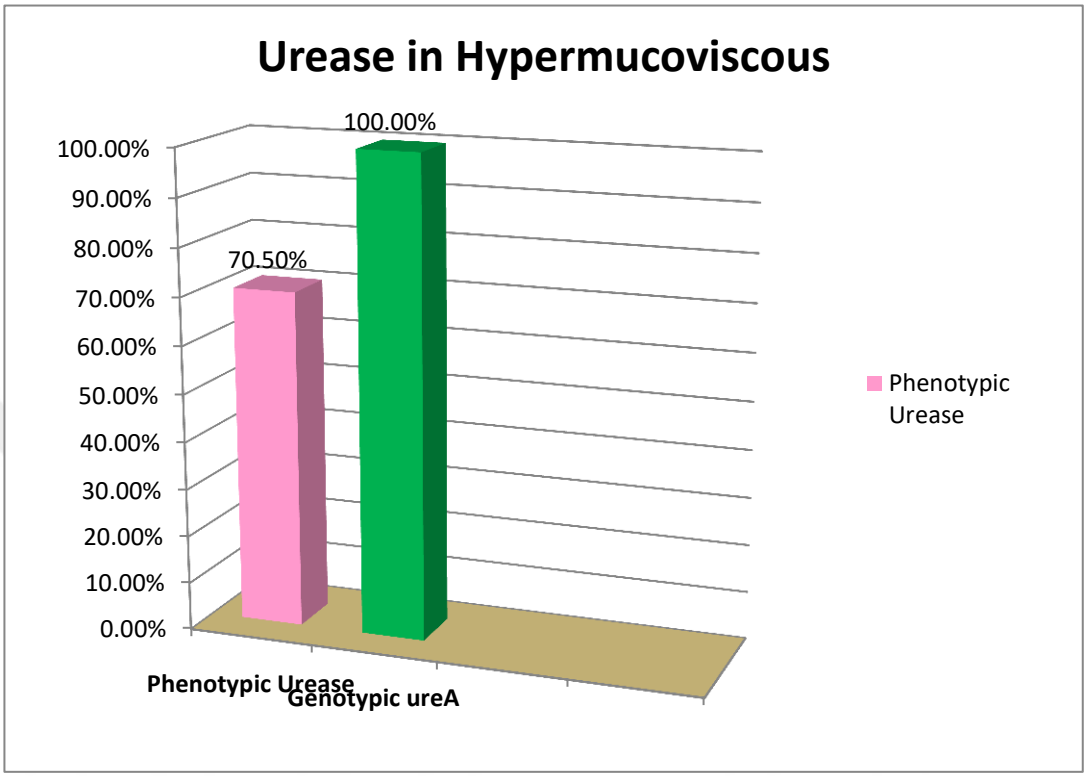

Figure 4.8 Comparison between the results of phenotypic and genotypic detection methods of urease

Urease can be produced by spp. According to Cheng *et al.* (2018) Ure A gene was detected in most of the hypermucoviscous K. pneumoniae isolates (97.0%). This result is very close to that of this study (100%).

Urease activity causes the pH of human urine to rise, allowing struvite and carbonate apatite to precipitate from ordinarily soluble polyvalent ions. Urinary stones are produced by these chemicals aggregating around bacteria. Microorganisms are shielded from medicines and the host's immune system inside such stones.Urinary stones can obstruct catheters or the urethra, which can cause acute bacteriuria (Różalski *et al.* 2007).

5. CONCLUSIONS AND RECOMMENDATION

It may be concluded that hypermucoviscous *Klebsiella pneumoniae* is an emerging pathogene. This pathogene posess extra polysaccharide capsule offeres it further defence against human immunity. String test was effective in detecting this phenotype. Urease enzyme is a potent virulence factor causes seroius infections one of them is the urinary tract infections and urinary stones. Phenotypic method to detect urease production was less effective than the molecular technique. It may be the gene responsible for urease coding ure A can not be expressed in some conditions.

There is a great need to further studies to detect other virulence factors in hypermucoviscous *Klebsiella pneumoniae.*

Capsular polysaccharide, lipopolysacharide, proteases, and other factors must be studied by different methods to evaluate the differences between classical and hypermucoviscous *K. Pneumoniae*.

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APPENDICES

APPENDIX 1. Apparatus and equipment that used in this study are mentioned in table

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Apparatus	Company / Origin
Autoclave	Fisher/ Japan
Centrifuge	Germany
Deep freezer	Sanyo / japan
Electric balance	Precisa /Switzerland
Gel electrophoresis apparatus	Cleaver / UK
Incubator	Nave / Turkey
Light Microscope	Olympus / Japan
Micro spin Centrifuge	Hettich /Germany
Micropipette	Dragon-med / Spain
Master cycler Gradient PCR	Eppendorf / Germany
Oven	Memmert / Germany
pH meter	Hanna / Romania
Refrigerator	Beko / UK
UV Transilluminator documentation system	Ultra Violet products institute /USA
Vitek system	Biomerieuk / USA
Vortex	Memmert /Germany
Water Distiller	Germany
Water bath	USA

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