

**Virulence Determinants and Antibiotic Resistance of
Extended-Spectrum Beta-lactamase (ESBL) Producing
Klebsiella pneumoniae Isolated from Hospital Environment**

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial
fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
Fall, 2019

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Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

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ABSTRACT:

Klebsiella pneumoniae is a member of gram-negative Enterobacteriaceae associated with community and hospital-acquired infections through highly resistant clones. Infection by Carbapenem-resistant *Klebsiella* is increasing day by day at an alarming rate. Occurrence of nosocomial bacterial infections affecting neonates, elderly and immune-compromised people has been increased from 8% to 40% in recent times. Hyper-virulent *K. pneumoniae* causes community-acquired infections in healthy individuals. In this study, the isolates were collected from different hospital environments for the detection of virulence genes and to see the antibiotic resistance of those samples. Antibiotic susceptibility to beta-lactams was tested for a total of 39 clinical isolates from 115 *K. pneumoniae* samples of different hospitals in Bangladesh. In this study, *K. pneumoniae* showed resistant to Cefexime(100%), Azetronam(69%), Tetracycline(66%), Nalidixic acid(61%) and Cefepime(53%). Resistance to Imipenem and Meropenem was 15% and 35% respectively. The majority of *K. pneumoniae* isolates characterized in the present study were resistant to different groups of antibiotics. On the other hand, for the detection of virulence genes, multiplex PCR was done on two sets. One of them included *fimH*, *ureA*, *kfubc* and other other one contained *uge* and *wabG* primers. Among the 115 samples, 35 samples were selected randomly for virulence gene detection. About 33% of them exhibited *fimH*, about 25% *Uge* and 42% of them showed *wabG*. CR-KP organisms are resistant to all beta-lactams. *K. pneumoniae* strains possess a serious threat to the immunosuppressive patient. This study was to observe the current status of the antibiogram of carbapenem-resistant and extended-spectrum beta-lactamases (ESBL) producing *K pneumoniae* and the molecular detection of these organisms collected from the hospital environments. The outcome of this study also provided new insights into the pathogenesis and the resistance of *K. pneumoniae*.

Dedication

My beloved family members

Acknowledgment

First of all, I would like to thank GOD for blessing me with good health, wellbeing, strength and patience to carry out and complete this research work. I do express my heartfelt gratitude to all those who provided me the opportunity to complete this thesis paper. Special gratitude goes to **Professor A. F. M Yusuf Haider** (Chairperson of the Department of Mathematics and Natural Sciences, BRAC University), and Professor **Dr. Mahboob Hossain** (Coordinator of Microbiology Program, BRAC University). I remain indebted to BRAC university for giving me the chance to undertake my thesis project at International Center for Diarrhoeal Disease Research ,icddr,b.

I express my profound gratitude to **Dr. Zahid Hayat Mahmud**, Associate Scientist, Project coordinator and Head, Laboratory of Environmental Health, icddr,b for offering me the privilege to work at his lab and to interact with the eminent researchers and being trained under their supervision.

I would like to express my sincerest appreciation and honor to **MD.Sobur Ali** (Research Officer, Laboratory of Environmental Health, icddr,b) for his proper guidance, advice and all round supervision during my research work. I am beholden to him for his constant encouragement and suggestions.

I am obliged to all the senior supervisors, scientists, research officers, research assistants, staff members, employees and students of icddr,b who open-heartedly allowed me to use all the required equipment and necessary materials to complete the thesis specially I am very much grateful to my friend **Faria Marha** for her relentless support, care, inspiration and positivity towards my work.

Last but not the least, I owe to my family, for their prolonged patience and nourishment towards my achievement and I am immensely grateful to my parents, without whom I wouldn't be who I am today. Their love, endless encouragement and faith in me, made me stronger and motivated. I also would like to express my wholehearted love to my younger sister for her continuous support, care and inspiration.

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Ethical statement

This study has been conducted with samples from the Swab study performed by International Centre for Diarrheal Diseases Research, Bangladesh (icddr,b) and the consent was taken to use the samples for thesis purpose.

List of Acronyms

CR-KP: Carbapenem resistant *K. pneumoniae*

MDR : Multidrug resistant

PCR : Polymerase chain reaction

LPS : Lipopolysaccharide

UTI: Urinary tract infection

ESBL: Extended-spectrum beta lactamase

CRE: Carbapenem resistant *K. pneumoniae*

AST: Antimicrobial susceptibility testing

HGT: Horizontal gene transfer

PBS: Phosphate buffer solution

Chapter 1 Introduction

Klebsiella pneumoniae is one of the most common Enterobacteriaceae associated with the community- and hospital-acquired infections. In recent years, the misuse and overuse of antibiotics have given rise to the serious health problem worldwide (Nordmann, Naas, & Poirel, 2011), infections by Carbapenem-resistant *Klebsiella* is increasing with an alarming rate of treatment failure and mortality. (Temkin, Adler, Lerner, & Carmeli, 2014) *K. pneumoniae* infections related to a variety of circulating clones comprise of up to 8% of all nosocomial bacterial infections affecting neonates, the elderly, and the immune-compromised with mortality rates reaching as high as 40% (Nordmann et al., 2011) ESBL-producing Enterobacteriaceae including *K. pneumoniae* were listed as pathogens of critical priority for research and development of antibiotics by the World Health Organization in 2017(Shrivastava, Shrivastava, & Ramasamy, 2018).

Two major types of antibiotic resistance have been commonly observed in *K. pneumoniae*, one mechanism involves the expression of extended-spectrum beta-lactamases (ESBLs), which render bacteria resistant to cephalosporins and monobactams. The other mechanism of resistance, which is even more troubling, is the expression of carbapenemases by *K. pneumoniae*, which renders bacteria resistant to almost all available beta-lactams, including the carbapenems. ESBLs are plasmid mediating having TEM-1, TEM-2 and SHV-1 derived enzymes that confers resistance to penicillin and cephalosporin, etc. ESBL CTX-M enzymes are predominant worldwide, and five different groups of CTX-M including CTX-M-1-2-8-9-25 groups have been described in *K. pneumoniae* strains.(Kluytmans-van den Bergh et al.,2016) The carbapenem-resistant genes are KPC, NDM-1, VIM, IMP, OXA-48 which are responsible for the resistance without additional permeability defects these enzymes are encoded by genes in either chromosome or acquired mobile elements such as plasmids and transposons. Additionally, the upregulation of efflux pumps or loss of porins and the upregulation of ESBLs or AmpC β -lactamases can lead to carbapenem resistance (Brinkworth et al., 2015).

CR-KP isolates were usually multidrug-resistant accompanied by resistance to extended-spectrum cephalosporins, fluoroquinolones, and aminoglycosides. There were previous studies frequently described the co-prevalence of carbapenem resistance and extended-spectrum beta-lactamase (ESBL) production.(Chen et al., 2011) Recently, the co-

existence of 16S rRNA methylase, which can render Enterobacteriaceae resistant to aminoglycoside, has also been reported in KPC-producing pathogens.(Wu, Liu, Han, Sun, & Ni, 2010)The potential spread of multi drug-resistant *K. pneumoniae* would strongly limit the therapeutic options. To better combat CR-KP infections, it is essential to understand the composition and distribution of antibiotic resistance genotypes. In this study, we will conduct molecular detection of virulence factors and co-existence of resistance determinants in CR-KP isolated from the hospital environment. Previous studies reported about the carbapenem-resistant *K. pneumonea* from hospital patients where the environmental conditions of the hospitals were not clear. In our study, we focused on the overall environment of the hospitals and the patients.

Chapter 2: Literature review

2.1 Taxonomy and identification of *K. pneumoniae*

K. pneumoniae is a Gram-negative, rod-shaped, facultative anaerobic bacterium (Funke & Bernard, 2011). It belongs to the family Enterobacteriaceae and the genus *Klebsiella*. The genus was first described in 1885 and was named after Edwin Klebs, German pathologist. The species received the name for its ability to cause severe pneumonia.

Key characteristics for identification are fermentation of lactose, capsule production, lack of motility, no indole production, negativity in methyl red test, and typical pattern in decarboxylase assays (lysine +, arginine -, ornithine -). It can be difficult to differentiate among *K. pneumoniae*, *Raoultella planticola* and *Raoultella terrigena* based on conventional biochemical tests, therefore misidentification in a clinical microbiology laboratory is possible (Alves, da Silva Dias, de Castro, Riley, & Moreira, 2006), (Maatallah et al., 2014). Discrimination by molecular method PCR against species-specific genes helps to identify *K. pneumoniae* more accurately.

2.2 Pathogenesis and virulence determinants

K. pneumoniae is usually considered as an opportunistic pathogen, mainly infecting hospitalized patients with underlying medical conditions. However, it is capable of causing severe disease (primary liver abscess with or without metastatic complications) in otherwise healthy individuals. Such strains are often referred to as being hyper-virulent. According to the molecular Koch's postulates several virulence determinants were identified in *K. pneumoniae*. A number of factors contribute to virulence and pathogenicity in *K. pneumoniae* such as the capsular serotype, lipopolysaccharide, biofilm formation, fimbriae, antibiotic resistance and iron-scavenging systems and adhesions (Wasfi, Elkhatib, & Ashour, 2016). To date, there are four major classes of virulence factors that have been well characterized in *K. pneumoniae*. These virulence factors consist of the capsule, including the production of hyper capsule in HV strains; lipopolysaccharide (LPS); siderophores; and fimbriae, also known as pili (Paczosa, Fisher, Maldonado-Arocho, & Meccas, 2014).

2.2.1 Capsule

Strains of *K. pneumoniae*, particularly clinical isolates, frequently produce a viscous polysaccharide capsule. The biochemical complexity of these capsules gives rise to the production of strain-specific antigenic types of capsular material. The ability of specific *K. pneumoniae* capsules to inhibit or impair phagocytosis by host cells was reported over 20 years ago (Cryz, Fürer, & Germanier, 1984) HV *K. pneumoniae* strains produce a hyper capsule, also known as being hyper-mucoviscous, which consists of a mucoviscous exopolysaccharide bacterial coating that may contribute significantly to the pathogenicity of HV *K. pneumoniae* (Yu et al., 2006). Both classical capsule and HV hyper capsule are made up of strain-specific capsular polysaccharides termed K antigens (i.e., K1 and K2, up through K78) (Pan et al., 2008)The association of the capsule with the lipopolysaccharide (LPS) has been investigated in a K2 serotype in which the capsule is bound by an ionic interaction to the LPS through a negative charge of carboxyl groups on GalA (Fresno et al., 2006)It was demonstrated that capsules possessing the Man- α -2-Man sequence exhibited a greater binding and susceptibility to phagocytic cells, a phenomenon termed lectionphagocytosis (Athamna et al., 1991). Not only may the capsule influence susceptibility to phagocytosis by direct binding to these cells, but it has also been demonstrated that the composition of the capsule influences opsonophagocytosis. Overall, the most virulent strains of *K. pneumoniae* produce a capsule composed of saccharides that do not facilitate binding to phagocytic cells and therefore are more resistant to phagocytosis. In addition, these capsules also present as poor opsonins and evade phagocytosis by complement activation. Since there are a greater variety of capsular serotypes implicated in UTIs compared to disseminating *K. pneumoniae* infections, the role of capsules in mucosal infections is less clear and a few studies of UTI-causing strains have indicated that serotypes from a wide variety of capsular types are found. The role of these capsules in protecting against innate defense mechanisms in the urinary tract has not been investigated in detail, but these structures could play a role in neutralizing host factors in this environment.

2.2.2 Lipopolysaccharide

LPS is both a beneficial and hindrance for *K. pneumoniae* during infection, as it is an important virulence factor that protects against humoral defenses but also can be a strong immune activator. LPS, also known as endotoxin, is a major and necessary component of

the outer leaflet of the cell membrane of all Gram-negative bacteria. Although there is considerable variation in LPS structures among bacterial species, it is typically comprised of an O antigen, a core oligosaccharide, and lipid A. These components are encoded by genes in the *wb*, *waa*, and *lpx* gene clusters, respectively (De Majumdar et al., 2015). Unlike the 77 different K antigens that have been documented for *K. pneumoniae* capsule, there have been only 9 different O-antigen types identified in *K. pneumoniae* isolates, and O1 is the most common (Hansen et al., 1999)

2.2.3 Fimbriae

Fimbriae represent another class of *K. pneumoniae* virulence factors and are important mediators of *K. pneumoniae* adhesion. In *K. pneumoniae*, type 1 and 3 fimbriae are the major adhesive structures that have been characterized as pathogenicity factors. Four other adhesive structures have been noted for *K. pneumoniae*, including another fimbria called KPF-28, a non fimbrial factor called CF29K, and a capsule-like material (Darfeuille-Michaud et al., 1992).

2.2.4 Siderophore

The growth of *K. pneumoniae* in vivo requires the capture and utilization of iron for essential metabolic processes. Since iron is also required by host cells, in order to survive the bacteria must compete with the host for available iron that is frequently in limited supplies. Host iron-binding compounds, such as transferrin and lactoferrin, are strong chelators of elemental iron and *K. pneumoniae* has evolved to produce its own iron-binding systems to scavenge the necessary iron required for its survival. Excess iron in hosts' tissues increased bacterial pathogenicity Enterochelin is a molecule comprised of three phenolic rings that are involved in the uptake of ferric ions (Neilands, 1995). The ferrienterochelin complex is bound to an 81-kDa outer-membrane receptor that is found in most strains of both *K. pneumoniae* and *E. coli*. Aerobactin is a hydroxamate compound comprised of a citrate molecule with N6-hydroxyethyl lysine and is derived by the oxidation of lysine.

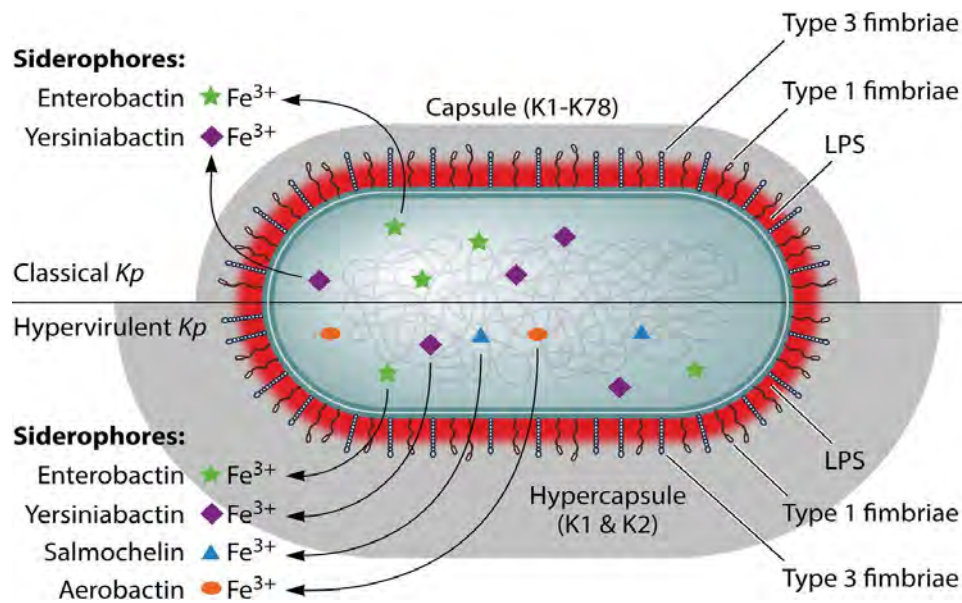


Figure: 1 Well-characterized virulence factors for pathogenic *K. pneumoniae*: capsule, LPS, fimbriae (type 1 and type 3) and siderophores.

2.2.5 Urease

Many strains of *Klebsiella* produce the extracellular enzyme urease that is a nickel-containing enzyme responsible for hydrolyzing urea to ammonia and carbamate. (Boer & Hausinger, 2012) The enzyme is assembled by the interaction of four accessory proteins (UreD, UreE, UreF, and UreG) involved in delivering nickel to the urease Apoprotein and one of these components (UreG) serves as a GTPase for activation. The role of urease production by *K. pneumoniae* in virulence and mediating the pathogenesis of the organisms, however, has yet to be demonstrated. Compared to species of *Proteus*, *K. pneumoniae* produces significantly less urease in vitro. In addition, it has been demonstrated that bacterially induced precipitation of salts onto abiotic surfaces in vitro by *K. pneumoniae* is less than that observed by urease-producing strains of *Proteus*. (Hedelin, Bratt, Eckerdal, & Lincoln, 1991) More recently, these results were confirmed by Broomfield and coworker (Broomfield, Morgan, Khan, & Stickler, 2009) who demonstrated that stains of *Klebsiella* were less efficient at forming encrustations and subsequently blocking catheters in a laboratory model of infection, compared to strains of *Proteus* and *Providencia*. Direct experiments to investigate the role of urease production by *K. pneumoniae* in vivo using an animal model of infection

have not yet been performed. However, the evidence to date indicates that this enzyme may only have a limited role in virulence by these bacteria.

2.3 Colonization in human and dissemination in hospitals

In humans, *K. pneumoniae* may colonize the skin, the naso- and oropharynx and the intestinal tract (ROSENTHAL & TAGER, 1975),(Thom, 1970). Carriage rates of *K. pneumoniae* are generally low in healthy humans but have been found to increase dramatically in hospitalized patients, especially in patients treated with broad-spectrum antibiotics (Rose & Schreier, 1968),(Davis & Matsen, 1974). The healthy human skin is not a common reservoir for Gram-negative bacteria, but *K. pneumoniae* has been found to colonize the hands of hospitalized patients and health care workers (HCWs) for several hours. (Casewell & Phillips, 1977),(Hart, Gibson, & Buckles, 1981) *K. pneumoniae* may spread remarkably well in the hospital environment, and frequently cause nosocomial infections and outbreaks, especially in intensive care units (ICUs). Medical equipment, the gastrointestinal tract of patients and the hands of HCWs are considered the most important reservoirs for the spread of *K. pneumoniae* in the hospital environment.(Casewell & Phillips, 1977), (Montgomerie, 1979). Moist surfaces and especially sink drains have been focused in several studies as a possible environmental source of transmission of Enterobacteriaceae, especially *Klebsiella* spp, to patients(Lowe et al., 2012). Although the environment was decontaminated and sinks were replaced, *K. pneumoniae* were recovered during further environmental screening, suggesting that these strains can survive well in that environment. Contamination of the hands of health care workers due to occasional backsplash during hand-washing in a contaminated sink and sink drains (Hota et al., 2009) or through moist surfaces near sinks and faucets has been suggested as a possible mode of transmission to health care workers and subsequently to patients in the ICU setting, facilitating low-frequent transmissions. Investigation revealed that several sinks were heavily contaminated by bacteria presumably from wastewater washed down the sink after cleaning of patients. A 'bed bath'-system for cleaning of patients were implemented to shortcut this opportunity.

2.4 Clinical manifestations

K. pneumoniae infections most often occur in hospitalized patients with different underlying medical conditions, but urinary tract infection, pneumonia or primary liver abscess might develop in healthy individuals in the community.

The most common nosocomial infections by *K. pneumoniae* are urinary tract infections (cystitis, pyelonephritis, renal and perirenal abscess), pneumonia 11 (bronchopneumonia, lobar pneumonia, bronchitis, ventilator-associated pneumonia), surgical-site infections and bloodstream infections (Suetens, Hopkins, Kolman, & Diaz Högberg, 2013). Besides the aforementioned clinical presentations *K. pneumoniae* can be involved in other pulmonary (acute exacerbation of chronic obstructive pulmonary disease, empyema) indwelling medical device-related (intravascular catheter, urinary catheter-related), intraabdominal (liver abscess, biliary tract infections, peritonitis) and central nervous system infections (post neurosurgical meningitis, brain abscess) as well (Bennett, Dolin, & Blaser, 2014). The infections can present as sporadic cases or as a part of an outbreak (Jarvis, Munn, Highsmith, Culver, & Hughes, 1985).

Pneumonia by *K. pneumoniae* is classically referred as Friedlander's disease, as it was considered to have special clinical features like: localization to the upper lobes, fissure sign on radiography, "currant jelly" sputum, propensity to develop an abscess and frequent occurrence in alcoholic patients. Nevertheless, etiologic diagnosis should not be based on the presence of these symptoms. (Bennett et al., 2014)

Primary liver abscess, sometimes with metastatic complications (bacteremia, meningitis, endophthalmitis, necrotizing fasciitis), is an emerging infectious disease caused by hypervirulent strains. It is observed mainly, but not exclusively in Asia (Siu, Yeh, Lin, Fung, & Chang, 2012).

2.5 Epidemiology

Since the mid-1980s, hypervirulent *K. pneumoniae*, generally associated with the hypermucoviscosity phenotype, has emerged as a clinically significant pathogen responsible for serious disseminated infections, such as pyogenic liver abscesses, osteomyelitis, and endophthalmitis, in a generally younger and healthier population. Hypervirulent *K. pneumoniae* infections were primarily found in East Asia and now are increasingly being reported worldwide. Although most hypervirulent *K. pneumoniae* isolates are antibiotic-susceptible, some isolates with combined virulence and resistance, such as the carbapenem-resistant hypervirulent *K. pneumoniae* isolates, are increasingly being detected. (Lee et al., 2017)

The first case of *K. pneumoniae* expressing a carbapenemase was identified in North Carolina in 1996, and thus, this type of carbapenemase is called KPC (Yigit et al., 2001). Regardless of the type of carbapenemase that they carry, carbapenem-resistant *K. pneumoniae* isolates are termed CRE for carbapenem-resistant Enterobacteriaceae. Due to a lack of available effective treatments, *K. pneumoniae* infections caused by ESBL-producing and carbapenem-resistant bacteria have significantly higher rates of morbidity and mortality than infections with nonresistant bacteria (Jacob et al., 2013)

2.6 β -lactam antibiotics

Antibiotics are antimicrobial compounds that either kill or inhibit bacterial growth. These compounds have different targets in the bacterial cell, depending on their mode of action. One of the most commonly used types of antibiotics is β -lactams. The β -lactam antibiotics have the β -lactam ring in common, while side chains are variable. There are four groups of β -lactam antibiotics: penicillins, monobactams, cephalosporins and carbapenems. (Kaspersen, 2015)

Mainly, β -lactams inhibit cell wall synthesis by binding to penicillin-binding proteins (PBPs), which are crucial enzymes in the cross-linking of peptidoglycan. Peptidoglycan is part of the cell wall of prokaryotes, and inhibition of peptidoglycan cross-linking will, as the cell grows, weaken cell integrity and eventually lyses the cell. Some β -lactam antibiotics have other modes of action in addition to the one described above. For example, cephalosporins might trigger autolytic enzymes in the cell envelope, causing cell lysis. Additionally, β -lactams have a different spectrum of activity, as some are broad-spectrum antibiotics with activity against both Gram-positive and -negative bacteria, while others have a narrow spectrum of activity, like the monobactams (which only affects aerobic Gram-negatives) (Kaspersen, 2015) Third and later generation cephalosporins have an extended-spectrum, which means they have an even broader range of activity.

Testing for susceptibility to antibiotics is called antimicrobial susceptibility testing (AST). An isolate that has low susceptibility to an antibiotic is said to be resistant. There is a variety of AST procedures available today. The disk-diffusion method is very common one, where agar plates and a disc with antimicrobial agents are used. Susceptibility is determined by the zone inhibition around each antibiotic disc. Commonly used automated AST-systems, such as Vitek 2, measure the minimum

inhibitory concentration (MIC), which is the lowest concentration (mg/L) of an antibacterial that is needed to inhibit bacterial growth.

2.7 Mechanisms of antibiotic resistance

Antibacterial resistance may be intrinsic (natural) or acquired. Intrinsic bacterial resistance to antibacterials, produced by other bacteria or fungi, existed in the environment before antibacterial compounds were taken into clinical use. Bacteria, furthermore, have the remarkable ability of environmental adaptation by changing their genome through mutations or by horizontal gene transfer (HGT), or by differential gene expression (Harbottle, Thakur, Zhao, & White, 2006). The extensive use of antibiotics in humans, but also in food animals and fish farming, has led to selective pressure in several environmental niches promoting the acquisition of resistance determinants. Resistance may be acquired in three ways: (i) mutations in chromosomal genes, causing altered antibacterial targets or transcriptional changes, (ii) acquisition of new genes by horizontal gene transfer (HGT), e.g. plasmid-mediated acquisition of β -lactamase encoding genes, (Jacoby & Sutton, 1991) or (iii) mutations in previously acquired genes, e.g. mutation in the β -lactamase genes blaTEM-1 and blaSHV1, resulting in production of enzymes with a broader spectrum β . (Jacoby & Sutton, 1991) Four biochemical mechanisms of antibacterial resistance have been described (Stein, 2005) (i) inactivation or modification of antibiotics by antibiotic-modifying enzymes, e.g. β -lactamases and aminoglycoside modifying enzymes (Azucena & Mobashery, 2001), (ii) modification of the target molecule (Hakenbeck & Coyette, 1998), (iii) restricted access to the target of an antibiotic due to reduction of porins in the outer membrane of Gram-negative bacteria, and (iv) efflux of one or more antibiotic groups from the bacterial cell due to efflux pumps in the cytoplasmic membrane.

2.7.1 Antibiotic-resistant of *K. pneumoniae*:

Two major types of antibiotic resistance have been commonly observed in *K. pneumoniae*. One mechanism involves the expression of extended-spectrum beta-lactamases (ESBLs), which render bacteria resistant to cephalosporins and monobactams. The other mechanism of resistance, which is even more troubling, is the expression of carbapenemases by *K. pneumoniae*, which renders bacteria resistant to almost all available beta-lactams, including the carbapenems. β -lactamases that harbors

weak carbapenemase activity in combination with membrane impermeability. (Nordmann, 2014) It is known that several carbapenemases including KPC, NDM-1 (Yong et al., 2009) VIM, IMP and OXA-48 were responsible for nonsusceptibility to carbapenems, without additional permeability defects (Pitout, Nordmann, & Poirel, 2015). These enzymes are encoded by genes in either chromosome or acquired mobile elements such as plasmids and transposons. KPC enzymes are currently the widest disseminated enzymes among *K. pneumoniae* isolates in Asia, America, and Europe. More than 20 different KPC variants have been identified, among which KPC-2 and -3 were the most common types (Chen et al., 2011). There were previous studies frequently described the co-prevalence of carbapenem resistance and extended-spectrum beta-lactamase (ESBL) production (Chen et al., 2011).

Chapter 3: METHODS

3.1 Sample collection

In this study, swab samples were collected from different hospitals of Bangladesh: Rajshahi medical, Rangpur medical and Faridpur medical. The samples were assembled from the patient and the hospital's environment around the patient: patient's bed, bed-pillow, floor, patient's hands as well as wounds and the caregiver's hand. Swab samples were transported in Phosphate Buffer Solution (PBS) containing 15 ml falcon tube, maintaining cool chain (4-10°C) to Laboratory of Environmental Health, International Centre for Diarrheal Research Bangladesh (icddr,b).

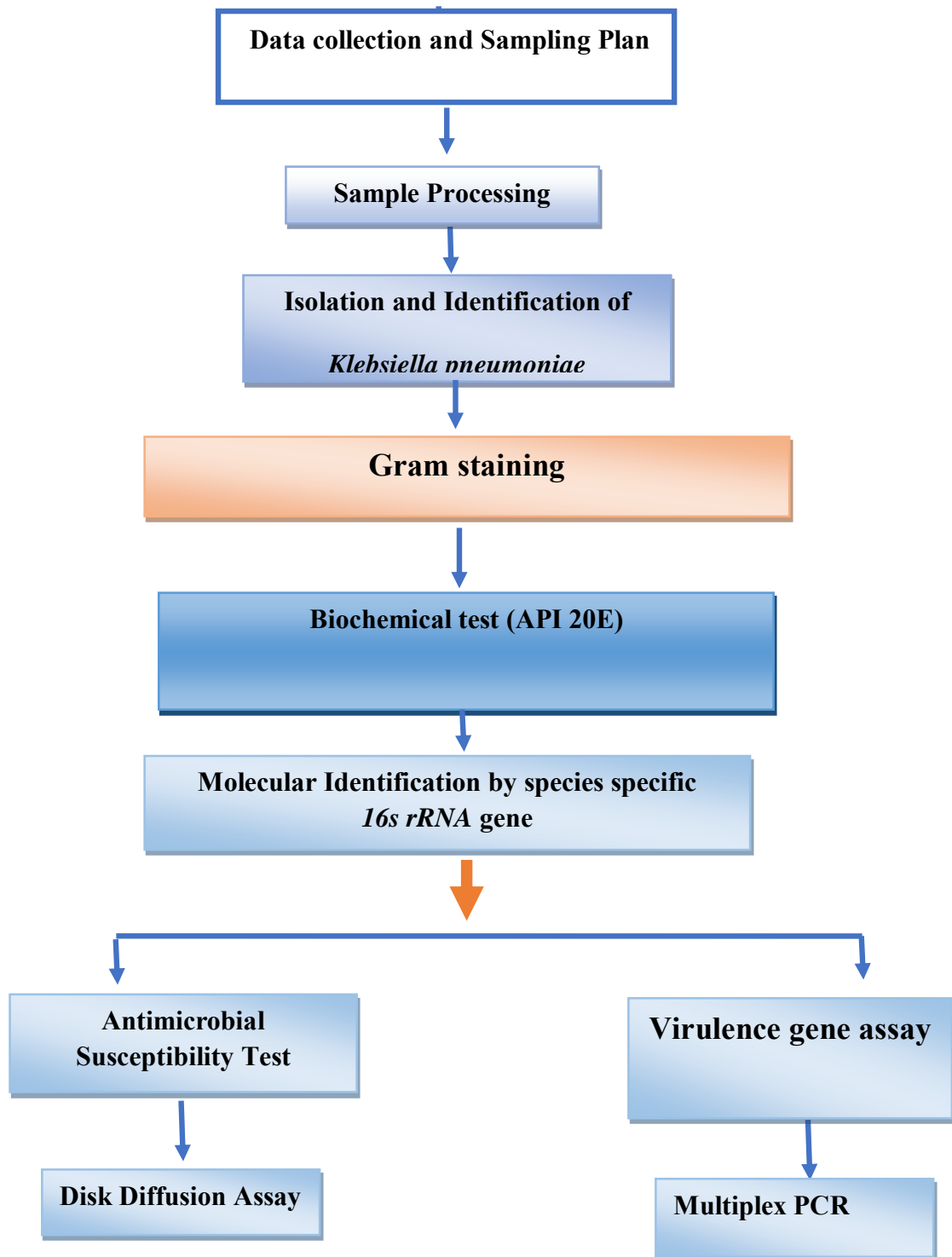
3.2 Sample processing

After receiving the sample in the Laboratory, Samples were placed to reach room temperature. After that, the swab samples along with the PBS were vortexed to homogenize the sample with the buffer. Then, 100 µl of the homogenized sample was spread on MacConkey Agar plate so that isolated colonies of bacteria that are evenly distributed in the plate and the plates were incubated at 37°C incubator for 24 hours.

3.3 Isolation and identification

3.3.1 Conventional method

Samples were spread on the MacConkey Agar plate and *Klebsiella pneumoniae* isolates were identified by colonies characteristics on the MacConkey Agar plate. After one hour of incubation in 37°C, the colonies were taken and streaked onto MacConkey.



3.3.2 Gram-Staining

For gram staining grease-free, a dry, sterilized slide was taken and a loopful of fresh suspected *K. pneumoniae* colony was smeared at the center of the slide. Then the smear was dried on the air and fixed by passing the slide 3-4 times through the flame quickly with the smear side facing up. The slides were then stained with crystal violet and washed with water and again the slide was flooded with gram iodine and left for 1 minute. The slides were then washed again and stained with safranin. Finally, the slides were placed under the microscope for observation.

3.3.3 Biochemical test (API 20E test)

Analytical profile index 20 E is a biochemical panel identification and differentiation of members of the family Enterobacteriaceae. It consists of 20 micro tubes containing dehydrated bacterial media. During incubation, metabolism produces color changes.

3.3.3.1 Preparation of the strip

An incubation box (tray and lid) was prepared and 5 ml of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere. The sample ID was recorded on the elongated flap of the tray. Then the strip was placed in the incubation box.

3.3.3.2 Preparation of the bacterial suspension

Different fresh single colonies of *K. pneumoniae* (from different pure culture samples) were picked up and using each of the 5 ml of bacterial suspensions were prepared using sterile 0.85% test tube.

3.3.3.3 Inoculation of the strip

A single well isolated fresh colony was taken using a sterile loop and carefully emulsified to achieve a homogeneous bacterial suspension. Immediately after preparation, with a pipette, the bacterial suspension was distributed into the tubes of the strip carefully avoiding bubble formation. For the CIT, VIP and GEL tests both tubes and cupules were filled. For the other tests, only the tubes (and not the cupules) were filled. For the test ADH, LDC, ODC, H₂S and URE anaerobiosis environment were created by overlaying the compartments with mineral oil. After that, the incubation box was closed and incubated at 37°C for 18-24 hours.

3.3.3.4 Reading the strips

The strips were read by referring to the reading table.

3.4 Screening of Carbapenem-resistant bacteria by patch inoculation on KPC CHROMagar and ESBL CHROMagar plate

CHROMagar KPC and CHROMagar ESBL is a commercially prepared chromogenic solid medium supplemented with agents that inhibit the growth of carbapenem-sensitive and ESBL negative bacteria respectively. Fresh single colonies of *K. pneumoniae* from the MacConkey agar plate were inoculated on CHROMagar KPC and CHROMagar ESBL agar plate by patch inoculation method. Then the plates with proper labeled were incubated at 37°C for 24 hours.

3.5 Molecular Identification by Polymerase Chain Reaction (PCR)

3.5.1 Culture preparation

Fresh pure bacterial culture was inoculated on MacConkey agar one day before the DNA extraction. The media plates were prepared and dried properly. The samples were streaked on the plates in the biosafety cabinet for safe handling. The plates after streaking were kept in the incubator at 37°C, for 24 hours.

3.5.2 DNA Extraction

The collected *K. pneumoniae* cells from culture plates were suspended in 600 µl of autoclaved distilled water and mixed thoroughly. The suspension was then boiled at 100°C for 10 min and then immediately placed on ice for 10 min. Upon centrifugation at 13000 rpm for 10 min, 100 microliter of the supernatant, containing the bacterial DNA was used as template in PCR, which was kept at -20°C before being assayed.

3.5.3. Molecular Confirmation of Suspected *K. pneumoniae* isolates by specific PCR

Isolates confirmed by the biochemical tests were further analyzed for the presence of 16srRNA gene which is a species-specific gene for *K. pneumoniae* detection.

Table 2.1: primer specific to 16srRNA gene of *K. pneumoniae* along with the annealing temperature, sequence and band size

Primer name	Sequence	Amplicon size	Reference	Annealing temperature
<i>16srRNA</i>	F-ATGTCGCAAGACCAGAGTGG R-GCACAACCTCCAAATCGACA	657	Sameya et al 2010	58

Table 2.2: reaction mixture for the PCR of *16srRNA* gene

Serial no	Components	Volume (μ l)
1.	2x Goteq green PCR master mix	12.5
2	Forward primer	1
3	Reverse primer	1
4	Nuclease free water	9
5	Template DNA	1.5
	Total Volume	25

A master mix was prepared for all isolates using the amounts mentioned above. After mixing the reaction mixture with the template DNA, the PCR tube containing reaction mixture was capped and then placed in a thermal cycler (Bio-Rad, Japan)

3.5.3.1 PCR condition for *16srRNA* gene

PCR for 16srRNA gene-specific primer was done by following these conditions (Sameya et al.2010) mentioned below in a thermal cycler.

Table 2.3 : PCR conditions for *16srRNA* gene

PCR steps	Temperature	Time Duration
Initial denaturation	94°C	1 min
Denaturation	94°C	10sec
Annealing	58°C	5sec
Extension	60°C	4min
Final extension	60°C	10 min
CYCLES	25	

3.5.3.2 Post PCR detection of amplified DNA by electrophoresis

The successful amplification of the gene is examined by resolving the PCR products in 1.5% agarose gel. 1.05g agarose (Sigma) was dissolved in 50 ml of 0.5X Tris-borate EDTA (TBE) buffer to give a final concentration of 1.5% agarose and was heated to dissolve in a microwave oven for about 2 min. When the temperature came down to 50°C, the gel was poured onto the gel tray already fixed with appropriate combs. Following solidification of the gel, it was submerged in 0.5X TBE buffer in a gel running tank. 7µl PCR products were loaded into the slots of the gel. Electrophoresis was continued with 80 volts until the dye migrates about 5-6cm from the wells (about 1.5-2.0 h). The gel was finally removed carefully and placed in the staining (EtBr) tray and stained for 15 minutes. Then the gel was de-stained for about 15 minutes in deionized water. The EtBr stained DNA bands were observed on a UV trans illuminator. Photographs were taken and bands were analyzed with Quality One ® software (Bio-Rad, USA). PCR product sizes were estimated using 100pb DNA size markers (Invitrogen, USA).

3.6 Antibiotic susceptibility testing

Randomly 35 confirmed *K. pneumoniae* were selected to evaluate their antibiotic-resistant pattern. The antibiotic discs used in this study were:

- nalidixic acid (30 µg) ,
- sulphamethoxazole/ trimethoprim (25 µg),
- gentamicin (10 µg),
- tetracycline (10 µg),
- ciprofloxacin (5 µg)
- cefepime (20 µg)
- azetronam (30 µg)
- cefixime (5 µg)
- imipenem (10 µg)
- meropenem (10 µg)

3.6.1 Inoculums preparation

According to the standard guideline by the Clinical and Laboratory Standard Institute (CLST) 0.5 McFarland turbidity standard was prepared. The preserved *K. pneumoniae* isolates were inoculated on nutrient agar plates and incubated overnight at 37°C. At least 2-3 well-grown colonies were selected from the nutrient agar plate and transferred into Mueller- Hinton Broth (MHB) using a sterile loop. Each tube containing 5ml MHB was incubated at 37°C after inoculation for 3-4 hours to achieve the 0.5 McFarland standards. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to the point of the 0.5 McFarland standards. *Escherichia coli* was used as a control.

3.6.2 Inoculation of Test Plates

Within 15 minutes of adjusting the turbidity of test culture, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the respected culture tube above the culture to remove the excess culture from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire surface of the plate. This procedure was repeated by streaking two more times rotating the plate approximately 60 degrees each time to ensure an even distribution. As a final step the rim of the agar was swabbed. The procedure was carried out under laminar air flow to avoid contamination. The lid was left open for 3-5 minutes but no more than 15 minutes, to allow for any access surface moisture to be absorbed before applying the drug impregnated disks.

3.6.3 Reading plates and result interpretation

After 16-18 hours of incubation, each plate was examined for the zone of inhibition, uniformly circular within a confluent lawn of growth. The diameters of the zones of complete inhibition (judged by unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of

inhibited growth, was ignored. The sizes of the zones of inhibition were interpreted by referring to the zone diameter interpretive standard from NCCLS2000.

3.7 PCR detection of virulence-associated genes

Two multiplex PCR was used to amplify five genes that have previously been associated with virulence in *K. pneumoniae*. These genes include those encoding for adhesions type I fimbriae (*fimH*), α -subunit of the urease, invasin related (*ureA*), iron acquisition system-related gene (*kfubc*), endotoxin-related genes (*wabG*, *uge*). Target genes, primers used and specific annealing temperature of PCR are given in table 2.4

Table 2.4 PCR primers for *K pneumoniae* virulence genes assays

Genes	Primer name	Primer sequence	PCR product size	Reference of primers	Annealing temperature
<i>kfubc</i>	kfuB-F1179	5'- GAA GTG ACG CTG TTT CTG GC -3'	797	Ma et al., 2005	55°C
	kfuC-R649	5'- TTT CGT GTG GCC AGT GAC TC -3'			
<i>fimH</i>	fim-1	5'- GCT CTG GCC GAT AC(C/T) AC(C/G) ACG G -3'	423	Brisse et al. 2009	55°C
	fim-2	5'- GC(G/A) (A/T)A (G/A)T AAC G(T/C) GCC TGG AAC GG -3'			
<i>ureA</i>	ureA-F	5'- GCT GAC TTA AGA GAA CGT TAT G -3'	337	Brisse et al. 2009	55°C
	ureA-R	5'- GAT CAT GGC GCT ACC T(C/T) A -3'			
<i>uge</i>	uge-F	5'- GAT CAT CCG GTC TCC CTG TA -3'	534	Regue et al., 2004	53°C
	uge-R	5'- TCT TCA CGC CTT CCT TCA CT -3'			
<i>wabG</i>	wabG-F	5'- CGGACTGGCAGATCCATATC - 3'	683	Brisse et al. 2009	53°C
	wabG-R	5'- ACCATCGGCCATTTGATAGA - 3'			

3.7.1 Multiplex PCR for *fimH*, *urea*, *kfubc*

One multiplex PCR was carried out to detect adhesions type I fimbriae gene (*fimH*), α -subunit of the urease, invasin related gene (*ureA*) and iron acquisition system-related gene (*kfubc*). A reaction mixture 25 μ l was prepared for this multiplex PCR as followed.

Table: 2.5 Multiplex PCR primers for *K pneumoniae* virulence genes assays (*fimH*, *urea*, *Kfubc*)

Serial No.	Components	Volume (µl)
1.	Master mix	12.5
2.	<i>fimH</i> forward primer	1
	<i>fimH</i> reverse primer	1
3	<i>ureA</i> forward primer	1
	<i>ureA</i> reverse primer	1
4.	<i>kfubc</i> forward primer	1
	<i>kfubc</i> reverse primer	1
5.	Autoclaved deionized water	5
6.	Template DNA	2
	<i>Total Volume</i>	25µl

Multiplex PCR for *fimH*, *urea*, and *kfubc* genes were done by following these conditions mentioned below in a thermal cycle:

Table: 2.6 Multiplex PCR conditions for *K pneumoniae* virulence genes assays (*fimH*, *urea*, *Kfubc*)

PCR steps	Temperature	Time Duration
Initial denaturation	94°C	5 min
Denaturation	94°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	1 min
Final extension	72°C	1 min
Cycle No	35	

3.7.3 Multiplex PCR for *wabG* and *uge*:

Another multiplex PCR was carried out to identify endotoxin-related genes *wabG* and *uge*. A reaction mixture 25 μ l was prepared for this multiplex PCR as followed.

Table 2.7: PCR reaction mixture for Multiplex PCR of *wabG* and *uge*

Serial No.	Components	Volume μ l
1.	Master mix	12.5
2.	<i>wabG</i> forward Primer	1
	<i>wabG</i> reverse primer	1
3.	<i>ugeF</i> forward Primer	1
	<i>ugeF</i> reverse primer	1
4.	Deionized water	6.5
5.	DNA template	2
	total	25 μ l

Multiplex PCR for *wabG* and *uge* genes was done by following these conditions mentioned below in a thermal cycle

Table 2.8: multiplex PCR condition for *wabG* and *uge* genes

PCR steps	Temperature	Time
Initial temperature	94°C	5min
Denaturation	94°C	30sec
Annealing	53°C	30sec
Extension	72°C	1 min
Final extension	72°C	1 min
Total Cycle	35	

PCR products were then analyzed by gel electrophoresis on 1.5% agarose gels in 0.5X TBE buffer. After staining in Ethidium bromide (EtBr), imaged using the Gel Doc (Bio-

Rad, USA) capture system. PCR product sizes were estimated using 100pb DNA size markers (Invitrogen, USA).

Chapter:4 Results

4.1 Isolation in MacConkey Agar

Samples that were streaked on selective media MacConkey Agar plate, showed pink mucoviscous colonies of *K. pneumoniae* (Figure 2)



Figure 2: MacConkey agar culture plate inoculated with experimental strains showed pink colonies with viscous/mucoid appearance.

4.2 Gram staining

The experimental bacteria showed the pink color of safranin stain suggesting that these were Gram-negative rod as shown in figure 3.

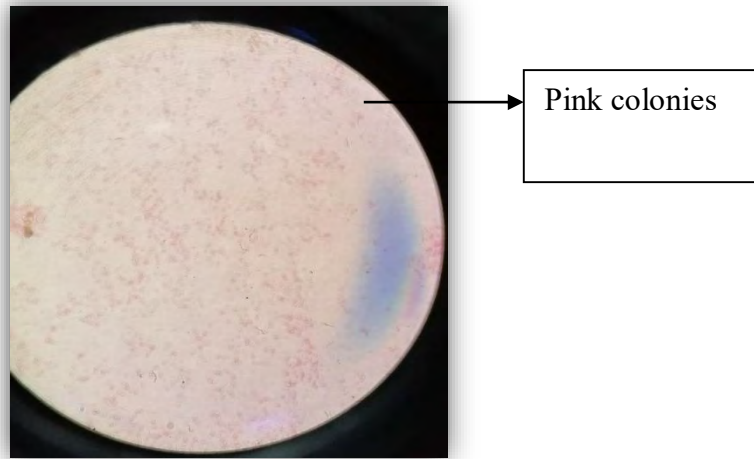


Figure 3: Bacteria shows pink colour colonies under a microscope indicating gram-negative rods

4.3 API20E test:

Among all of the isolates tested, the majority of isolates belonged to biochemical profile '5215773'. All isolates were identified as *K. pneumoniae* by a biochemical test using API20E. the result was interpreted by the colour change of biochemical testes after 24 hours of incubation.



Figure 4: After 24 hours incubation biochemical identification of *K. pneumoniae* by API20E kit

4.4 Detection of *K. pneumoniae* by PCR:

For the detection, *16srRNA* primer was used to detect the gene *16srRNA*. Among the 115 samples, 89 samples exhibited bands at 657 which means they were positive for the *16srRNA* gene and is almost 77% of them. The samples were collected from the patient's hand, bed rail, the floor of the hospitals, caregiver's hand and bed pillow.

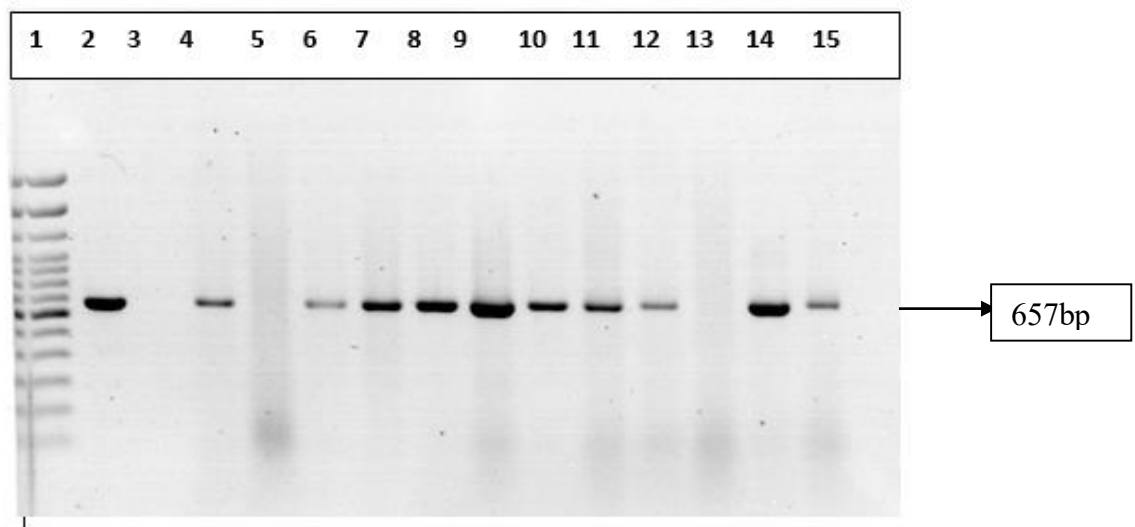
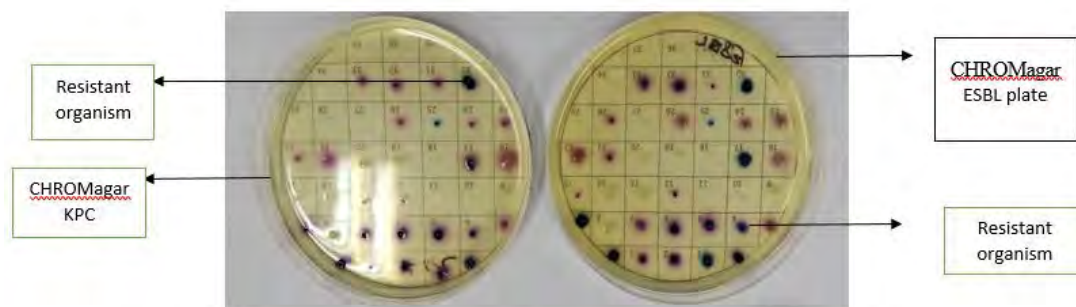


Figure 5: Bands at 657 ,from the left lane ,lane 1:ladder, Lane :2: positive control ,Lane:3 negative control ,Lane 4: FPMSBPS-11-FH-5 Lane6 : FPMSBPS-11-FH-4 ,Lane 7: FPMSBPS-11-FH-2 , Lane :8FPMSBPS-11-FH-1,Lane :9 FPFMBNT-14-FL-1 Lane10: FPFMBNT-14-FL-2 ,Lane :11 FPFMBNT-14-FH-1, Lane:12 FPFMBNT-14-PH-2, Lane:14 FPPNNBNT-13-BP-3Lane:15: FPPDBNT-11-BP

4.5 ESBL producing *K. pneumoniae*

From 120 environmental samples tested (patient's hand, bed rail, the floor of the hospitals, caregiver's hand and bed pillow), 35 samples were found to be contaminated with ESBL positive *K. pneumoniae*. From these, a total number of 115 *K. pneumoniae* isolates were isolated on CHROMagar ESBL. After incubating at 37°C for 24 hours they gave metallic blue colors on the ESBL plates. To avoid isolates duplication, from 115 isolates 35 were selected as representative *K. pneumoniae* from 35 contaminated samples. From these 40% were

form floor, 20% from bed pillow, 9% from both care givers hand and bed rail. These isolates were further investigated for their ability to produce carbapenemase enzyme in KPC Chromagar. We found 45% (n=16) isolates to produce metallic blue colony on KPC Chromagar. Among the samples that were screened as carbapenem-resistant by patch inoculation was found mainly on the floor (about 50%), bed pillow 25% and bed rail 6.25%. The samples screened as having both ESBL producing and carbapenem-resistant *K. pneumoniae* showed greater presence at floor samples (about 53%) in bed pillow 20% and bed rail 6.60%.



4.6 Antibiotic Resistance Pattern

The results of antimicrobial susceptibility testing of the 39 strains randomly chosen from the samples are shown in Table 4.5. A high prevalence of resistance to azetronam (69%) was observed. Resistance to tetracycline (66%) gentamycin (41%), Most of the isolates were susceptible to quinolone and fluoroquinolone antimicrobials including ciprofloxacin (48%), nalixic acid (61%). Some isolates were resistant to extended-spectrum cephalosporins like cefotaxime cefixime (100%) and cefepime (53%) and carbapenem antibiotics like imipenem (15%) and meropenem(43%).

Table :2.9 Antibiotic susceptibility of *K. pneumoniae* strains.

Name of antibiotic	Resistant	Intermediate	Susceptible
Monobactam			
Azetronam	69%	0%	30%
Tetracyclin			
Tetracyclin	66%	0%	33%
Aminoglycosides			
Gentamycin	41%	2%	59%
Fluroquinolone			
Ciprofloxacin	48%	2%	48%
Quinolone			
Nalidixic acid	61%	10%	28%
Extended spectrum cephalosporin			
Cefixime	100%	0%	0%
Cefepime	53%	2%	43%
Carbapenem			
Imipenem	15%	28%	51%
Meropenem	43%		53%
Sulphomethoxazole/trimethoprim			53%



Figure 6: Antibiogram by disk diffusion method using muller Hinton agar (MHA)

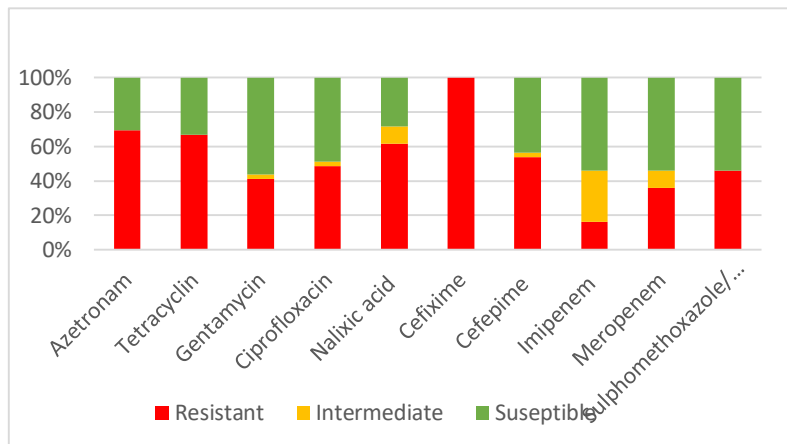


Figure 7: Resistance and susceptibility percentage of different isolates of *K. pneumoniae* samples against different antibiotics

Table:2.10 Multidrug resistance *K.pneumoniae*

Sample ID	Numbers of groups	Name of the antibiotics
RSNNBN T-13-FL		Azetronam, tetracyclin, Gentamycin, Ciprofloxacin, Cefixime, Cefepime, Meropenem.
RSNNBN T-14-BP	5	Azetronam, tetracyclin, Gentamycin, Ciprofloxacin, Cefixime, Cefepime, Imipenem Meropenem
RSPDBN T-12	3	Azetronam, Tetracyclin, Gentamycin, Ciprofloxacin, Cefixime, Cefepime, Imipenem Meropenem.
RSPDBN T-12	5	Azetronam , Tetracycline, Gentamycin
RPPDBN T-11-BR	7	Azetronam,Tetracyclin,Nalidixicacid,Cefexime,Sulphomethoxazole/trimethoprim
RSPDBN T-11-BR	7	Azetronam,Tetracyclin, Gentamycin Nalidixic acid,Imipenem,Meropenem , Sulphomethoxazole/trimethoprim
RSMSBP S-14FL	4	Azetronam,Tetracyclin, Gentamycin Nalidixic acid,Imipenem,Meropenem ,Sulphomethoxazole/trimethoprim
RSMSBP S-13-BP	6	Azetronam ,Cefepime ,Cefixime,Sulphomethoxazole/trimethoprim
FPMMB NT-11-FL	7	Azetronam ,Tetracyclin, Gentamycin Nalidixic acid,Imipenem,Meropenem ,Sulphomethoxazole/trimethoprim

4.8 Virulence gene detection by multiplex PCR

For detection of virulence genes multiplex PCR was done on two sets. Set 1 included *fimH* , *ureA*, *kfubc*, set two contained *uge* and *wabG* primers. Among the 115 samples, 35 samples were selected randomly for virulence gene detection. About 33% of them exhibited *fimH*, about 46% *uge* and 77% of them were found to be positive for *wabG*. (Figure 4.8)

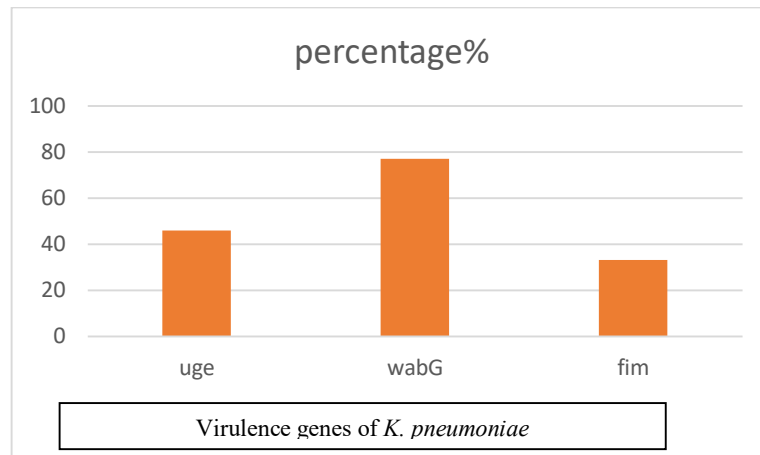


Figure : 8 the percentage of virulence genes *fimH*, *uge* and *wabG*. Here *fimH* was present in 20 samples out of 35 and the percentage is 33%, *wabG* was present in 27 out of 35 samples which is almost 77% and *uge* was present in 16 out of 35 samples almost 46%

Most of the samples contained *wabG* gene. The presence of *fimH*, *uge* and *wabG* virulence genes were confirmed by multiplex PCR and they showed bands at 423,534 and 683.*fimH* gene was multiplex along with *kfubc* and *ureA* primers but *kfubc* and *ureA* didn't show bands .that means among the samples these two genes were not present. Among 35 samples 11 of them showed the *fimH*, *wabG*, and *uge* virulence genes expect *kfubc* and *ureA*.

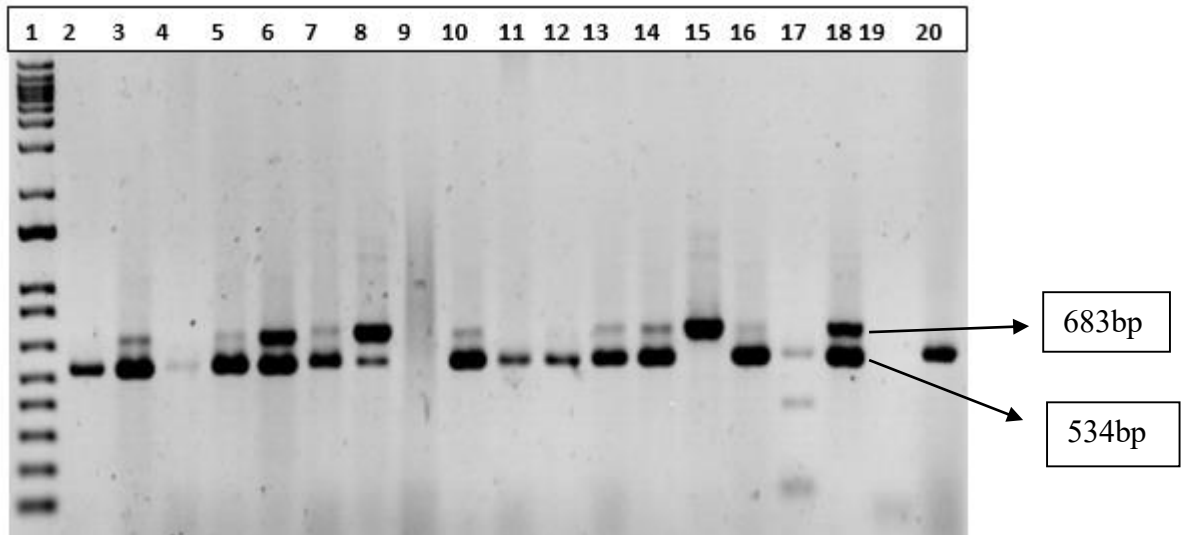


Figure 9: *wabG* at 683,from left lane, L:1 is ladder, L:2positive control ,L3:RSMMBNT-12-FL,L5:RSMMBNT-11-FLL7:RSNNBNT-12-BP-7,L14 :RSNNBNT-14-BP-4,L15:RSMSBPS-14-FL-1,L119: RPPDBNT-13-BR-3 *uge* at 534,from left Lane 1:ladder,L2:positive control,L3: RSMMBNT-12-FL,L:5RSMMBNT-11-FL,L6: RSNNBNT-12-BP-7,L7: RSNNBNT-14-BP-1,L8: RSNNBNT-14-BP-4,L10: RSPDBNT-14-FL-2,L11: RSPDBNT-13-1,L12: RSPDBNT-11-BR-1,L13: RSPDBNT-12-1,L14: RSPDBNT-12-3,L16: RSMSBPS-13-BP-1,L18: RPPDBNT-13-BR-3.

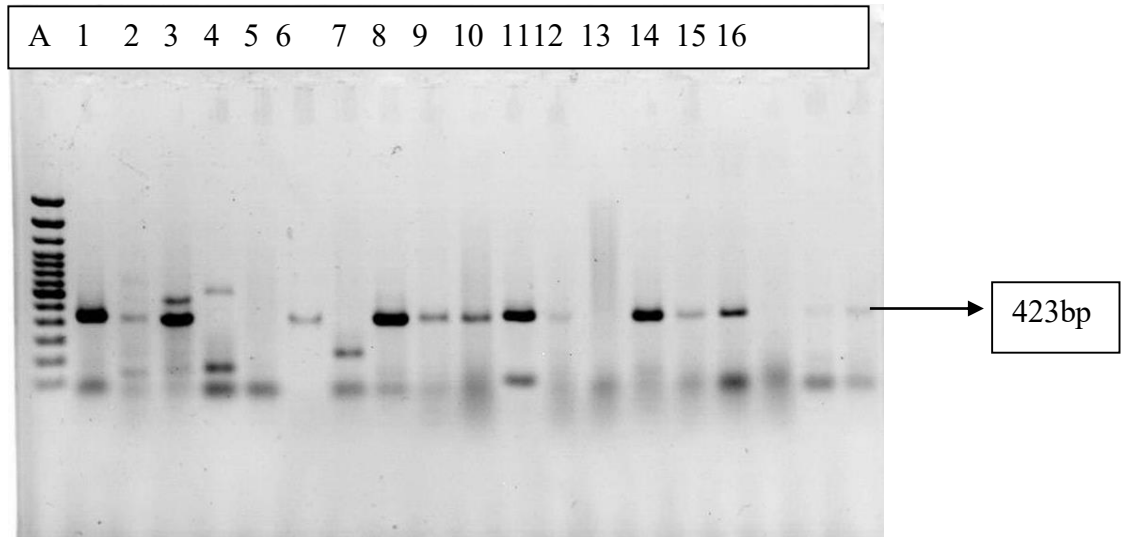


Figure 10: *fimH* at 423,L1: positive control ,L2: RSPDBNT-14-FL-2,L3: RSMSBPS-13-BP-1,L8: RPPDBNT-14-FL,L9:RPPDBNT-11-BP-1,L:10 FPFMBNT-14-FL-1,L:11 FPFMBNT-14-FL-2,L14: FPMMBNT-11-FL-4,L15: FPMMBNT-11-5,L16: FPMMBNT-11-3

Among all the virulence genes found from 35 environmental samples, 14 of them were found from the floor samples (FL) which is about 40% of the total samples,7 of them found from the bed pillow(BP) ,3 of them from caregiver's hand(FH) and 3 of them found from bed rail (BR)sample. It has been shown on a table

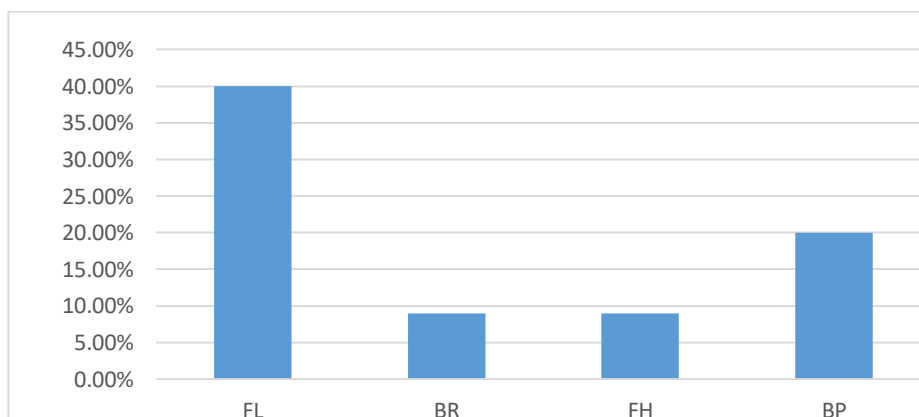


Figure : 11 percentage of virulence genes found from the different area of hospital environment, FL=floor,PH=patients hand, FH=patients care giver hand, BP=bed pillow, BR=bed rail.

5. Discussion

K. pneumoniae is an important cause of multidrug-resistant infections worldwide. Epidemiological studies have highlighted the emergence of multidrug-resistant and virulent *K. pneumoniae* strains isolated from hospital-acquired infections (Beceiro, Tomás, & Bou, 2013; Wasfi, Elkhatib, & Ashour, 2016). Antibiotic-resistant and virulent bacteria caused more severe diseases for longer periods of time. The antibiotic resistance of *K. pneumoniae* strains is associated mainly with the production of ESBL. In 2017 the World Health Organization included ESBL-producing *K. pneumoniae* in the list of the most dangerous superbugs along with *Acinetobacter baumani* and *Pseudomonas aeruginosa* (Shrivastava, Shrivastava, & Ramasamy, 2018).

The present research was done to assess the prevalence, distribution of virulence genes phenotypic analyses of antibiotic resistance amongst the *K. pneumoniae* strains isolated from hospital environment. In this study, out of 120 samples tested, 32% (n=39) were contaminated with ESBL producing *K. pneumoniae*. Isolates from these samples showed 100% resistance to third-generation cephalosporin (3GC). There are many possible contributing factors to the emergence, and spread of ESBL producing *K. pneumoniae* in hospital environment, including the new acquisition of resistance genes; transfer of antibiotic resistance genes; healthcare exposure; use of indwelling medical devices; and inappropriate and excessive antibiotic use in health care (Ayukekbong, Ntemgwa, & Atabe, 2017).

K. pneumoniae has carbapenemases the enzymes capable of breaking down most β -lactams including carbapenems, and confer resistance to these drugs. Reports indicate that carbapenemase-producing enterobacteriaceae isolates seem to be increasing in number in the last few years. In our study, we found 42% carbapenemase producers from KPC Chromagar plate. From AST result, we found 33% isolates were resistant to imipenem and 15% were resistant to meropenem which is similar to a previous report (Aseem, Shenoy, Mala, Baliga, & Ashish, 2016). Resistance to these last-resort antibiotics can be attributed to the lack of strict policies and unprescribed use of antibiotics in Bangladesh (Ahmed, Rabbi, & Sultana, 2019). Resistant to azetronam, tetracycline and nalidixic acid were more predominant in *K. pneumoniae* 69%, 66%, 61% respectively. The majority of *K. pneumoniae* pathogens characterized in our study

were resistant isolates to different groups of antibiotics. We found about 26% (n=10) of the ESBL-*K. pneumoniae* were resistant multidrug-resistant which implies that not only β -lactam antibiotics but resistance to other classes of antibiotics is being co-selected. This is a great concern, as the samples were taken mainly from the environment of the hospitals. This is a noticeable fact because after surgery or any medical treatment this opportunistic *K. pneumoniae* can cause disease not only to the immune-compromised people and patients but also to the healthy human being as well. Before prescribing a new antibiotic, physicians should be suggested to reserve as 'last line' drugs to combat serious infectious.

Recent reports have shown that *K. pneumoniae* strains can accumulate, increasing their pathogenicity and causing severe and difficult-to-treat infections. Adhesive organelles *fimHI* involved in urinary tract infections was documented by (Struve, Bojer, & Krogfelt, 2008) in this, Type 1 fimbriae, *fimHI* was detected in half of the ESBL- *K. pneumoniae*. An earlier study reported that *wabG* gene is associated with invasive and serious infections but their mechanism in disease development is still unclear.(Turton, Perry, Elgohari, & Hampton, 2010) Mutant *wabG* gene produced non-capsulated and less virulent *K. pneumoniae* strains in murine pneumonia model. Mutant *wabG* gene produced defective core polysaccharides and was unable to induce urinary tract infection that proved an important role in the pathogenicity of *K. pneumoniae* infections.(Izquierdo et al., 2003) In this study, the *wabG* gene was found about 77% which is quite similar with a previous study where they documented that about 87.5% of the isolates harbored *wabG* gene (Aljanaby & Alhasani, 2016).

Previous findings reported that laboratory animals harboring *K. pneumoniae* strains without *uge* were less virulent than strains with *uge* gene (Regué et al., 2004). We found, 45% (n=16) of *K. pneumoniae* to harbor *uge* gene which is similar to the previous study ((Regué et al., 2004) . In this study, we found most of the virulent isolates having the virulence genes(*fim H*, *wabG*, and *uge*)from the patient's caregiver's hand ,bed rail and floor samples which is a concerning fact because the hospital environment can be a fact of infection caused by *K. pneumoniae*. Due to time constrain we failed to characterize the drug-resistant genes and biofilm study of the samples we got. But, this study will help to understand the composition and distribution of antibiotic resistance genotypes and will help us to have an idea about virulence and co-existence of resistance determinants in *K. pneumoniae* isolated from the hospital environment.

Conclusions

K. pneumoniae has become of increasing concern in the clinical environment over the last few decades. The isolation of MDR strains causing nosocomial acquired infections and the relatively rapid spread of this resistance strains among strains has made the treatment and management of these types of infections difficult. The most common type of *K. pneumoniae* infection associated with hospitalized patients is pneumonia. As this antibiotic resistance of this organism is increasing before prescribing a new antibiotic, physicians would be suggested to reserve it for only the worst cases. It is also suggested to prescribe the old antibiotics by analyzing the resistance pattern. So, new antibiotics can be considered as 'last line' drugs to combat serious infectious illnesses. And the future management of these infections should be taken like including the development of non-antibiotic based therapies. The increased quality of healthcare has resulted in a greater population of susceptible hosts for *K. pneumoniae* infection. The prevention of infection and the management of patients with infections will provide enormous challenges in the future.

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