Phenotypic and genotypic characterization of hypervirulent *Klebsiella pneumoniae* isolated from neonates in Bangladesh

By

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A thesis submitted to the Department of Mathematics and Natural Sciences, BRAC University in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology

Microbiology Program
Department of Mathematics and Natural Science
BRAC UNIVERSITY
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Declaration

I hereby declare that the thesis entitled **" Phenotypic and genotypic characterization of hypervirulent *Klebsiella pneumoniae* isolated from neonates in Bangladesh"** submitted by me, **Qurratul Ain** to the Department of Mathematics and Natural Sciences, BRAC University is a record of original and independent research work carried out by me under the supervision of **Fahim Kabir Monjurul Haque, PhD** from BRAC University and **Senjuti Saha, PhD** from the Child Health Research Foundation while completing degree at BRAC University. I further declare that this thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing. This thesis has not been submitted previously, in part or in full, to any other university or institution for any degree or other qualification. I have acknowledged all main sources of help.

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Approval

The thesis titled "Phenotypic and genotypic characterization of hypervirulent *Klebsiella pneumoniae* isolated from neonates in Bangladesh" submitted by Qurratul Ain (19326036) in Summer, 2024 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 5 September 2024.

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Ethics Statement

The thesis titled "Phenotypic and genotypic characterization of hypervirulent *Klebsiella pneumoniae* isolated from neonates in Bangladesh" is based on research involving patient samples and data collected from Bangladesh Shishu Hospital and Institute and the Child Health Research Foundation (CHRF). Ethical approval for this study was obtained from the Institutional Review Board (IRB) of BRAC University. Strict confidentiality protocols were maintained to ensure patient privacy. Identifiable patient information was anonymized, and data were securely stored and accessed only by authorized personnel involved in the research. All efforts were made to protect the confidentiality and anonymity of the participants. The research adhered to all relevant ethical guidelines and regulations, ensuring that the study was conducted ethically and responsibly. Every measure was taken to maintain the highest standards of integrity and ethical conduct throughout the research process.

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Dedication

I warmly dedicate this thesis to:

My beloved parents, whose endless love, unwavering support, and countless sacrifices have been the bedrock of all my accomplishments. Your guidance and encouragement have shaped my journey, and for that, I am eternally grateful.

To my loving sister, for always being there with immense support and motivation. Your belief in me has been a great source of strength.

Above all, to Almighty Allah, for granting me the strength, wisdom, patience, and perseverance to see this work through to completion. I am deeply thankful for the countless blessings and guidance that have illuminated my path.

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Acronyms

HvKp	Hypervirulent *Klebsiella pneumoniae*
CKp	Classical *Klebsiella pneumoniae*
CHRF	The Child Health Research Foundation
MDR	Multi-drug resistant
XDR	Extremely drug-resistant
ST	Sequence Type
CC	Clonal Complex
icddr,b	International Center for Diarrhoeal Disease Research, Bangladesh
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide BLAST
NCBI	National Center for Biotechnology Information
WGS	Whole Genome Sequence
PLA	Pyogenic Liver Abscess
KPC	*Klebsiella pneumoniae* carbapenemases
CDC	Centers for Disease Control and Prevention
AMR	Antimicrobial resistance
CLSI	Clinical and Laboratory Standards Institute
DORB	Discharge On Risk Bond
DSH	Dhaka Shishu Hospital
SSF	Shishu Shastho Foundation
CMOSH	Chattogram Ma-O-Shishu Hospital
VFDB	Virulence Factor Database

Abstract

A systematic assessment of 11 observational studies reported that 596 (42.8%) of 1392 *Klebsiella pneumoniae* infection cases were hypervirulent (hvKp) strains. Additionally, a research study conducted in Bangladesh reported that 19% of their *Klebsiella pneumoniae* isolates were hvKp strains. Unlike usual *Klebsiella pneumoniae* infections, hvKp strains increasingly affect healthy people, making them more vulnerable. To understand gene variations and the presence of hvKp strains, datasets from NCBI were retrieved for bioinformatics analyses. Following that, the hvKp strain was identified by BLAST and Kleborate on 50 CHRF *Klebsiella pneumoniae* isolates. Two isolates with hypervirulent genes were discovered to have virulence scores of 4 and 5. Out of 19 samples, the string test was positive for 3 samples, and all of them exhibited multi-drug resistance. Moreover, it was discovered that all three patients were diagnosed with sepsis or neonatal sepsis following the detection of meningitis. Additionally, clinical manifestations revealed that a total of four patients were diagnosed with meningitis (one fatal case), and three with pneumonia or severe pneumonia. Subsequently, antibiotic susceptibility tests showed that over 41% of isolates were resistant to 13 antibiotics, with limited treatment options. Finally, the findings highlight the variations in hypervirulent gene sequences and suggest that the current virulence gene database is not comprehensive. Therefore, a repository for hvKp serotypes should be developed, and the database should be updated frequently.

Keywords: *Klebsiella pneumoniae;* Hypervirulent; meningitis; neonatal sepsis; multidrug resistance; antibiogram;

CHAPTER 1
INTRODUCTION

1. Introduction

1.1. *Klebsiella pneumoniae* infection

Klebsiella pneumoniae is well-known as an opportunistic pathogen, accounting for the second most prevalent cause of invasive infections in humans, such as bacteremia [1]. Among various *Klebsiella* species, *Klebsiella pneumoniae* is responsible for 70% of human infections [2]. It is a non-motile, gram-negative, encapsulated nosocomial pathogen that infects immunocompromised individuals and neonates [3]. It can be found in soil, surface water, and on medical devices [4]. In humans, *Klebsiella pneumoniae* infects blood and other organs via the gastrointestinal system and nasopharynx [5]. Moreover, it is inherently resistant to some antibiotics and is of risk to humans and infants. Due to limited treatment options, it is associated with high morbidity and mortality [6]. Furthermore, the rise of multidrug-resistant *Klebsiella pneumoniae* and hypervirulent *Klebsiella pneumoniae* (HvKp) is becoming increasingly problematic, particularly given its rapid global dissemination [5].

1.2. Antibiotic Resistance of *Klebsiella pneumoniae*

Klebsiella pneumoniae is a pathogen of concern due to its extensive antibiotic resistance [6]. During the 2000s, various global surveillance investigations were conducted that found that 20-80% of *Klebsiella pneumoniae* isolates were resistant to first-line antibiotics such as cephalosporins, fluoroquinolones, and aminoglycosides [2]. According to several estimates, the fatality rate for bloodstream infection (BSI) ranges from 15-79% due to multi-resistant *Klebsiella pneumoniae* [7]. There have been reports of two forms of antibiotic resistance in *Klebsiella pneumoniae*. The first process includes the production of extended-spectrum β-lactamases (ESBLs), which cause bacteria to become resistant to cephalosporins and monobactam antibiotics. The second method involves the production of carbapenemase, which makes practically all available β-lactamases and carbapenems resistant. Since the discovery of the carbapenemase enzyme, *Klebsiella pneumoniae* carbapenemases (KPCs) have become a global concern [8]. Furthermore, over the last 15 years, a strain of KPC-carrying *Klebsiella pneumoniae*, ST258 (Sequence Type) and CC258 (Clonal Complex), has spread around the world, increasing the frequency of infection cases [9].

The Child Health Research Foundation (CHRF) conducted a study at Bangladesh Shishu Hospital and Institute and discovered that children under the age of five were infected with carbapenem-

resistant invasive *Klebsiella pneumoniae*. This is frightening because carbapenem resistance increased from 21% to 65% in 342 isolates over the course of 6 years. In addition, antibiotics with high consistency showed gradual resistance. For example, ampicillin, ceftriaxone, cotrimoxazole, and ciprofloxacin, have resistance rates of 80-100%, whereas amikacin and chloramphenicol have resistance rates ranging from 39% to 80% [10]. Furthermore, a study was conducted at the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b) [11] and Dhaka Medical College [12] on the antibiotic resistance of *Klebsiella pneumoniae*, which revealed that *Klebsiella pneumoniae* are resistant to majority of bactericidal and bacteriostatic agents. Figure- 1 displays the percentage of antibiotic-resistant *Klebsiella pneumoniae* in Bangladesh.

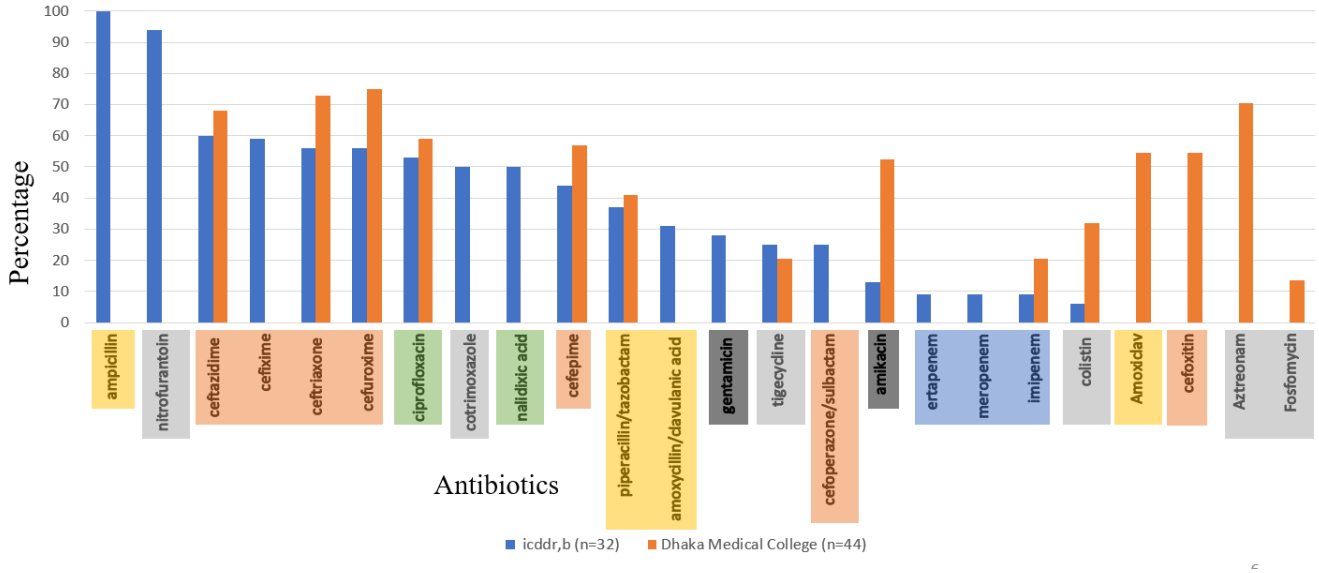

Figure-1: Antibiotic resistance of *Klebsiella pneumoniae* in Bangladesh (2022-2023).

Patients infected with KPC-producing *Klebsiella pneumoniae* had a greater mortality rate than the general population [13]. Furthermore, in 2013, the Centers for Disease Control and Prevention (CDC) reported on a survey undertaken in 2011 to assess the incidence and severeity of these strains. It was reported that 183 hospitals in the United States had ESBL-producing strains that caused 23% of nosocomial infections, resulting in 1,100 fatalities from 17,000 illnesses. In contrast, the carbapenemase-producing strain was responsible for 11% of nosocomial infections, with 520 deaths recorded from 7,900 illnesses [9]. However, despite efforts by researchers to unravel the virulence and pathogenic mechanisms of *Klebsiella pneumoniae*, the mechanism of

virulence and antibiotic resistance remains elusive. As a result, the growth of hypervirulence and multidrug resistance has been a major concern in recent years. [5]

1.3. Hypervirulent *Klebsiella pneumoniae*

Hypervirulent *Klebsiella pneumoniae* is a serious pathogen that infects healthy people and causes life-threatening illnesses [14]. Historically, *Klebsiella pneumoniae* was most likely to infect immunocompromised persons. However, the recent appearance and spread of hypervirulent strains is infecting healthy and immune-sufficient individuals while also increasing the number of people who are susceptible to infections [9]. According to Kawser and Shamsuzzaman (2022), infections caused by hypervirulent strains, although antibiotic-sensitive, are associated with increased morbidity and mortality. *Klebsiella pneumoniae* is classified into two pathotypes based on its phenotypic appearance and genotypic markers: classical *Klebsiella pneumoniae* (CKp) and hypervirulent *Klebsiella pneumoniae* (HvKp). Figure- 2 highlights the key distinctions between CKp and HvKp, as well as a comparison of MDR-HvKp [15].

Characteristics	Classical *K. pneumoniae* (cKP)	Hypervirulent *K. pneumoniae* (hvKP)	Multidrug-resistant hvKP (MDR-hvKP)	References
Infections	**Acquisition:** nosocomial **Host:** immunocompromised patients **Geographic region:** the whole world **Infectious sites:** urinary tract infections, pneumonia, bloodstream infections; usually polymicrobial at sites of infection **Metastasis:** uncommon	**Acquisition:** community **Host:** healthy adults **Geographic region:** Southeast Asia **Infectious sites:** pyogenic liver abscess, meningitis, endophthalmitis, necrotizing fasciitis; usually monomicrobial at sites of infection **Metastasis:** Common	**Acquisition:** nosocomial and community **Host:** usually immunocompromised patients **Geographic region:** Asia (especially China) **Infection sites:** pyogenic liver abscess, bloodstream infections, urinary tract infections	Harada et al., 2019; Russo and Marr, 2019; Liu C. et al., 2020; Tang et al., 2020
Phenotypes	Non-hypermucoviscosity and string <5 mm	Hypermucoviscosity and string ≥5 mm	Hypermucoviscosity or non-hypermucoviscosity	Russo et al., 2018
Common serotypes	K1–K79	K1, K2, K5, K16, K20, K54, K57, KN1	K1, K2, K16, K20, K54, K62, K64, K47	Pan et al., 2008, 2015; Yang et al., 2021
Siderophores	Enterobactin, yersiniabactin	Enterobactin, yersiniabactin, salmochelin, and aerobactin	Enterobactin, yersiniabactin, salmochelin, and aerobactin	Russo et al., 2015; Lam et al., 2018b; Choby et al., 2020

Figure-2: Comparison of CKp, HvKp, and MDR-HvKp. (Source: Zhu, J., et al., *Virulence Factors in Hypervirulent Klebsiella pneumoniae.* Frontiers in Microbiology, 2021. **12**.).

Hypervirulent *Klebsiella pneumoniae* can infect hospitalized hosts as well as healthy individuals from the community with severe infectious disorders. Hypervirulent *Klebsiella pneumoniae* differs from other Enterobacteriaceae members in severeal ways, including its ability to infect healthy people of any age, its ability to produce infections at various places, and its later development of metastatic dissemination. Moreover, the unique clinical condition of this strain is that it develops

hepatic abscesses without biliary tract disease. Unfortunately, hvKp can result in infections practically anywhere in the body, including meningitis, pneumonia, necrotizing fasciitis, endophthalmitis, and non-hepatic abscesses [16]. Furthermore, Xu et al. (2019) have revealed that, since 2014, a substantial proportion of KPC-2-producing hvKp-causing meningitis has been observed, which is likely to cause serious infection, particularly among patients with post-neurosurgical meningitis. Figure- 3 represents and compares the infection site of both classical and hypervirulent *Klebsiella pneumoniae.*

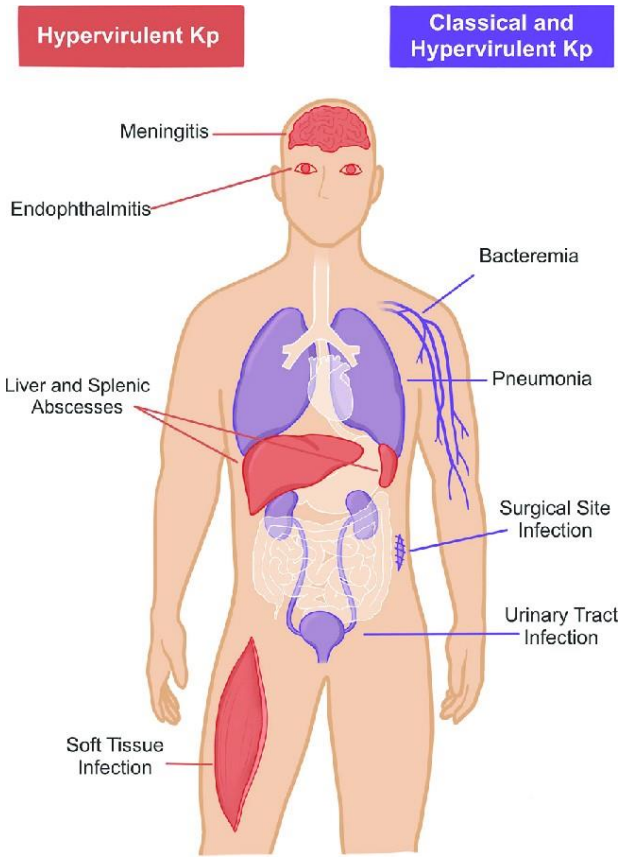

Figure-3: Anatomical sites of Hypervirulent *Klebsiella pneumoniae* and Classical *Klebsiella pneumoniae* infection. (Source: Gonzalez-Ferrer, S., et al., *Finding Order in the Chaos: Outstanding Questions in Klebsiella pneumoniae Pathogenesis.* Infection and Immunity, 2021. **89**.).

Additionally, some early reports claimed that regularly used antibiotics might treat previously hypervirulent *Klebsiella pneumoniae* strains. However, it was recently revealed that hypervirulent *Klebsiella pneumoniae* isolates from the present are exhibiting multidrug resistance due to

acquired resistance determinants [17]. Furthermore, 596 (42.8%) of the 1392 *Klebsiella pneumoniae* infection cases were found to be hvKp strains, according to a systematic assessment of 11 observational studies. This finding explains the severity of the illness produced by the strain [18]. Additionally, studies conducted by the International Center for Diarrheal Disease Research, Bangladesh (icddr,b) revealed that 6 isolates (19%) out of 32 *Klebsiella pneumoniae* isolates were HvKp strains. The hypervirulent *Klebsiella pneumoniae* was identified using a custom database of 28 *Klebsiella pneumoniae* virulence genes (*fimD, fimK, fimH, fimC*, *mrkD, mrkJ, mrkF, mrkC, mrkA, mrkI, iutA, entB, ybtS, iucA, ybtA, irp1, irp2, fyuA, p-rmpA, p-rmpA2, iroB, iroC, iroD, iroN, alls, pilW, clpV/tssH, peg-344,* and *pks* gene cluster) collected from the Virulence Factor Database (VFDB) and known virulence genes from NCBI. BLASTn analysis was used to determine the presence of the virulence genes. If the isolates had at least one recognized biomarker from the custom database, including *peg-344, iroB, iucA, rmpA,* or *rmpA2*, they were considered to be hypervirulent [11]. It is therefore necessary to determine the prevalence and infection rate of hvKp in Bangladesh, as this suggests that the pathogen has a high virulence score.

1.4. Detection process of Hypervirulent *Klebsiella pneumoniae*

The first occurrence of hypervirulent *Klebsiella pneumoniae* infection was discovered in a patient with a liver abscess in Taiwan [19]. Thus far, the virulence factors of hypervirulent *Klebsiella pneumoniae* have been classified into four main classes: the production of hyper-capsules, lipopolysaccharides, siderophores, and fimbriae [20]. Two prominent characteristics are primarily emphasized among them. First, a hypermucoviscous phenotype is caused by over-expression of *rmpA* and/or *rmpA2*. According to Huang et al. (2023), the second benefit is enhanced iron absorption via four siderophores: aerobactin (*iucABCD-iutA*), salmochelin (iroBCDEN), yersiniabactin, and enterobactin. To characterize the hypervirulent strain, however, different researchers are employing different biomarkers. Although the clinical symptoms of infection caused by the hvkp strain are well understood, only a limited number of biological indications may be used to distinguish the hvkps strain from other strains of *Klebsiella pneumoniae*. To comprehend the virulence of the hvkp strains, a thorough investigation and observation of the strains are necessary.

Prior studies have demonstrated that *rmpA* synthesizes capsular polysaccharides to regulate mucoid phenotype. This has been linked to 87.5% of *Klebsiella pneumoniae* strains that have been

related to pyogenic liver abscesses (PLA). Aerobactin, a dominating iron siderophore, was also thought to be a critical virulence factor. It was found in 93–100% of hvKp strains and was found in *Klebsiella pneumoniae* pyogenic liver abscesses. In a mouse lethality test, aerobactin increased virulence by a factor of 100. [21]. Most recently, it has shown to be quite accurate to distinguish between hvKp and cKp biomarkers using virulence plasmids. It has been reported that the siderophore assay and virulence genes such *rmpA*, *rmpA2*, *peg344, iroB,* and *iucA* can be utilized to identify hvKp. Moreover, strains with ≥30 μg/mL iron have been reported to be able to identify hvKp strains from cKp strains with >95% accuracy [20]. Additionally, the primary product of hypervirulent *Klebsiella pneumoniae* is aerobactin, which is encoded by the *iucABCD* operon. According to Kocsis (2023), over 90% of siderophore activity was reported in the examined strains, suggesting a high prevalence of hvKp. Furthermore, it has been noted that hvKp strains are more likely than non-hvKp germs to produce larger and more active iron-absorbing molecules, a sign of their pathogenicity and virulence [5]. A "string test" is also used to determine the hypermucoviscous phenotype of hvKp strains. The colonies are grown onto agar media such as Mueller-Hinton, Columbia blood agar, and MacConkey agar plates. The string test result is considered positive if a viscous string of > 5 mm in length forms for an incubation period of 36 ± 1°C and 24 hours under aerobic conditions [22]. Table-1 clearly illustrates the markers and virulence factors associated with hypervirulent *Klebsiella pneumoniae* and aids in strain identification [17].

Virulence Determinants	**Gene**	**Marker**
Hypermucoviscosity		capsular types: K1, K2, K5, K16, K20, K28, K54, K57, K63
		string test (> 5mm)
	magA	mucoviscosity associated gene
	rmpA	regulator of the mucoid phenotype a gene
	rmpA2	
Siderophore	*iucABCD*	aerobactin siderophore biosynthesis
	peg-344	putative transporter
	peg-589	putative carboxymuconolactone decarboxylase family

	iroB	salmochelin siderophore biosynthesis
	irp2	yerseniabactin siderophore biosynthesis
	ybt	yerseniabactin
Other	*terB*	tellurit resistance

Table-1: Markers and virulence determinants associated with hypervirulent *Klebsiella pneumoniae.* (Source*:* Kocsis, B., *Hypervirulent Klebsiella pneumoniae : An update on epidemiology, detection and antibiotic resistance.* Acta Microbiologica et Immunologica Hungarica, 2023. **70**(4): p. 278-287.)

1.5. Challenges while detecting the strain

The siderophore assay, string test, biofilm, and biomarkers for polymerase chain reaction (PCR) are used in the identification process of hvKp. However, to achieve therapeutic decision-making, early identification of hvKp is necessary. This is because many antibiotics are unable to get through the blood-brain and blood-CSF barriers, making it challenging to treat the infection [23]. Furthermore, not enough clinical information exists to compare infection with hvKp and cKp. It's crucial to understand how hvKp and *Klebsiella pneumoniae* compare in terms of MDR and XDR (extremely drug resistant) to detect hvKp quickly.

1.6. Putative findings after detecting the hypervirulent strain

Initially, the identification and characterization of hypervirulent *Klebsiella pneumoniae* would facilitate the study of the prevalence of HvKp genes from the circulating capsular serotypes of *Klebsiella pneumoniae* in Bangladesh. Furthermore, it will be possible to move forward with the development of a vaccine that is effective against strains of HvKp. Additionally, if a patient's symptoms resemble the illness brought on by the hvkp strain, PCR testing can be utilized to determine whether the patient is infected with that strain. The therapeutic decision-making process will consequently become easier. A patient may present with signs and symptoms such as endophthalmitis, liver or spleen abscess, meningitis, etc. The sample can be sent immediately for microbiological testing and PCR, anticipating hvkp. Early reporting of PCR results allows clinicians to make an informed decision and treat the patient with non-resistant antibiotics if the test results are positive. The PCR data provide an estimate of HvKP prevalence in Bangladesh. Ultimately, the development of a vaccination that is effective against HvKp strains and the identification of the antibiotic-resistant gene can prevent HvKp infection.

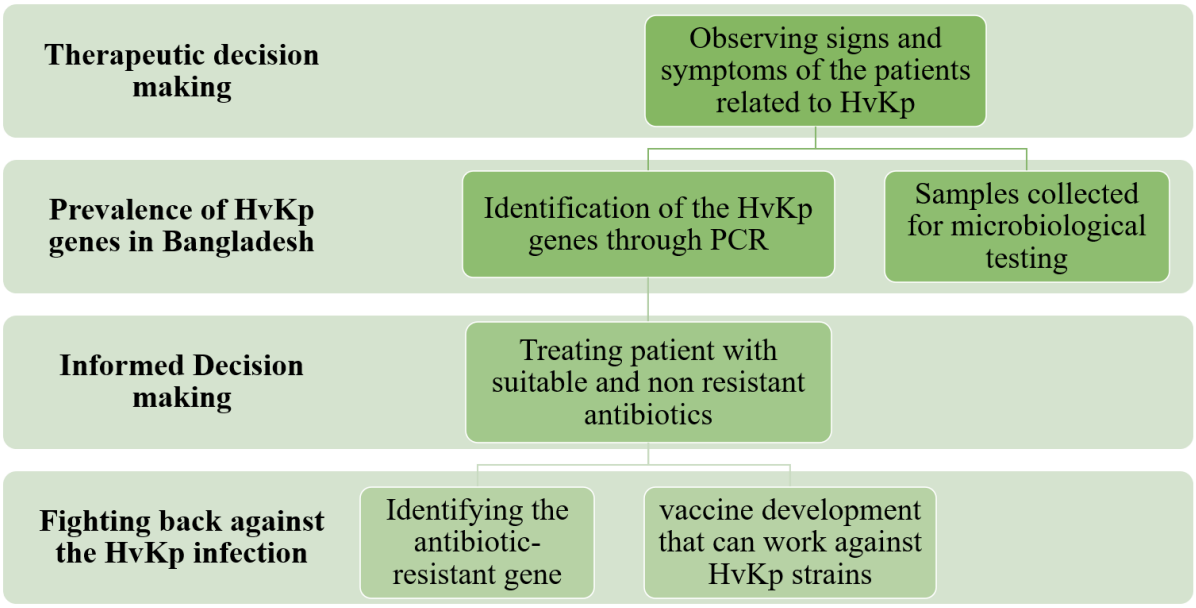

Figure-4: Implementation of the data outcome.

1.7. Objective

The objective of the study was to characterize hypervirulent *Klebsiella pneumoniae* (hvKp) using genotypic and phenotypic methods. In this regard, extensive bioinformatics was used to identify the virulence genes and laboratory tests were used to gain insights on phenotypic hypervirulence, To this end, a custom database and the software Kleborate were utilized to determine the virulence genes and virulence scores respectively.

CHAPTER 2
METHODOLOGY

2. Methodology

2.1. Bioinformatic pipeline for the identification of the strain

2.1.1. National Center for Biotechnology Information (NCBI)

In this study, the identification of hypervirulent *Klebsiella pneumoniae* was done from the complete genome sequenced data. Initially, the NCBI (National Center for Biotechnology Information) sequenced data were utilized to create a road map for finding the hvKp strains as a trial session. Firstly, the FASTA sequences of the virulence genes of *Klebsiella pneumoniae* were identified and retrieved. Among the virulence genes were *iutA, rmpACD, iucABCD, iroBCDN*, and *peg-344* from different strains of hvKp and the number of genes was 82. Moreover, 95 complete genome sequences were identified and their FASTA sequences were retrieved. Based on global reports of infections with multidrug-resistant and hypervirulent *Klebsiella pneumoniae* (MDR-hvKp) as of January 2021, a few selected countries including China, Japan, Taiwan, USA, and UK-were included in the data set [24]. The isolates from these countries were chosen because of the alarming number of HvKp infections in those countries. An NCBI filter search using 'hypervirulent *Klebsiella pneumoniae*' and 'country name' yielded the complete genome sequences. The sequences were retrieved because most of the sequenced genomes were submitted and published as hypervirulent *Klebsiella pneumoniae*. The isolates were sequenced and submitted by multiple institutions. The highlighted Table-2 shows the number of isolates from each institution, as well as their details.

Institutions	**Authors/ Submitted by**	**Year**	**Number of Isolates**
Taipei Veterans General Hospital and National Yang-Ming University	Lin-Xiang Wang	2017	7
National Yang-Ming University	Yi-Tsung Lin	2019	4
The Second Affiliated Hospital, University of South China	Logen Liu	2020	17
Liverpool School of Tropical Medicine	Daire Cantillon	2023	11
Hackensack-Meridian Health Center for Discovery and Innovation	Liang Chen	2021	27
National Yang Ming Chiao Tung University	Duong Tran	2024	18

Department of Bacteriology, Graduate School of Medicine, Osaka City University		2021	2
China-Japan Friendship Hospital	Jiankang Zhao	2021	9

Table-2: Details of 95 genome sequences retrieved from NCBI.

2.1.2. Basic Local Alignment Search Tool (BLAST)

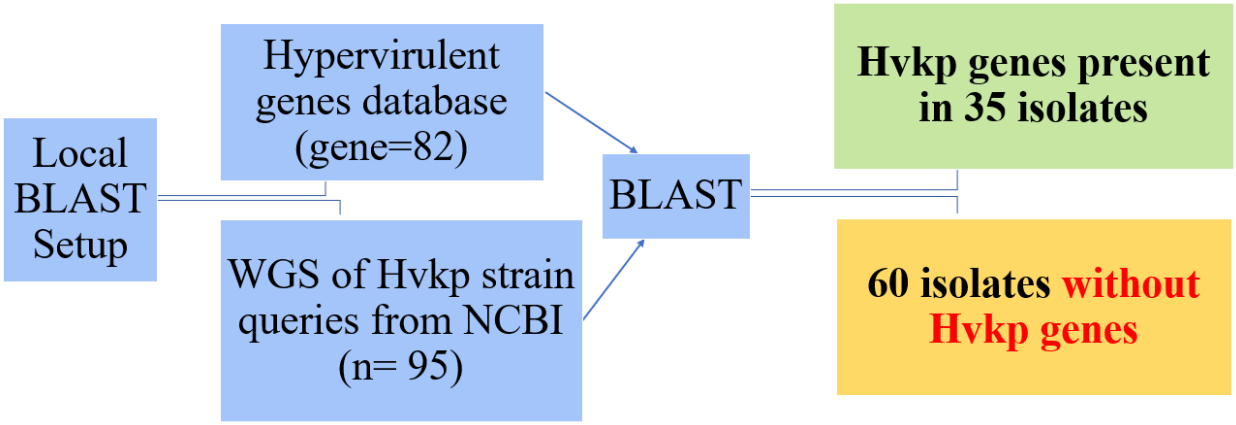

Figure-5: Identifying HvKp strain using BLAST.

Following the local installation of the Basic Local Alignment Search Tool (BLAST), a database containing 82 virulence genes from several hvKp strains was created. After that, 95 complete genome sequencing data sets were acquired from NCBI and used as BLAST queries. Following the BLAST run, the results revealed that 35 isolates had Hvkp genes while 60 isolates did not.

2.1.3. Kleborate

To further understand it, Kleborate was utilized, and the virulence score of the hvKp strain was employed to define it. Kleborate is a genotyping tool which was designed especially for *Klebsiella pneumoniae* and the associated species complex. It is used to find the virulence gene locus, virulence score, and antimicrobial resistance (AMR) determinants [25]. Afterward, 35 isolates with genes identified as hypervirulent were tested in Kleborate once more to determine and establish a threshold for virulence score.

Figure-6: Validating the process of identification.

The Kleborate score reference served as the basis for determining the virulence score. In this case, virulence score 3, or the presence of the aerobactin gene cluster (iucABCD), was regarded as the threshold. According to Lam et al. (2021), the virulence score in Kleborate is a range of 0 to 5, indicating the presence of virulence gene loci and its increasing risk (yersiniabactin < colibactin < aerobactin). For simplicity, it has been indicated that neither the *rmpADC* nor the salmochelin *(iro)* locus is specifically taken into account in the virulence score. The aerobactin (iuc) locus on *Klebsiella pneumoniae* virulence plasmids usually has the *iro* and *rmpADC* loci next to it. Therefore, the presence of *iuc* (score of 3-5) usually indicates the presence *of iro* and *rmpADC*. However, aerobactin was given priority in the score computation since it is more strongly linked to blood expansion and more indicative of the hypervirulence phenotype [26].

Virulence score	Details
0	negative for all of yersiniabactin (*ybt*), colibactin (*clb)*, aerobactin (*iuc*)
1	yersiniabactin only
2	yersiniabactin and colibactin (or colibactin only)
3	aerobactin (without yersiniabactin or colibactin)
4	aerobactin with yersiniabactin (without colibactin)
5	yersiniabactin, colibactin, and aerobactin

Table-3: Virulence Score of Kleborate. (Source: Lam, M. M. C., et al. (2021). "A genomic surveillance framework and genotyping tool for Klebsiella pneumoniae and its related species complex." Nature Communications **12**(1): 4188.)

Once a threshold was established, the identification was verified by combining local BLAST and Kleborate. For this reason, 95 complete genome sequences were once more utilized to calculate each isolate's virulence score. After 95 isolates were subjected to Kleborate analysis, 63 isolates

had virulence score of 0 or 1 and had no presence of hypervirulent genes. Nine of them had a Virulence Score of less than 3, and the presence of hypervirulent genes. On the other hand, 23 isolates had a Virulence Score of 3 along with hypervirulent genes.

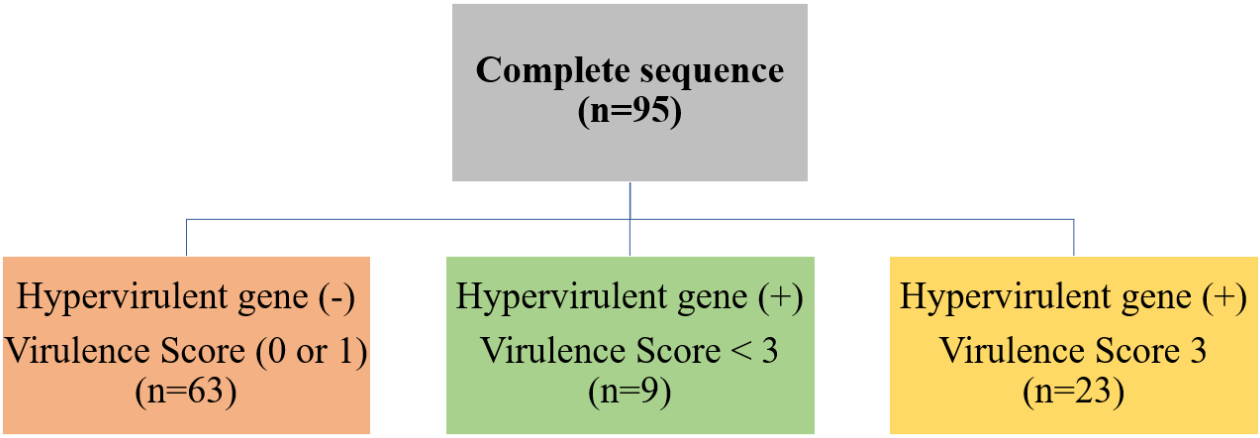

Figure-7: Final Validation to identify HvKp using Kleborate.

2.2. Sequenced Sample of Child Health Research Foundation (CHRF)

Following the validation procedure, the hypervirulent *Klebsiella pneumoniae* strain was identified by local BLAST and Kleborate on 50 CHRF *Klebsiella pneumoniae* isolates. It was noted that only two isolates with hypervirulent genes were discovered. The two isolates were also discovered to have virulence scores of 4 and 5. Coincidentally, the hypervirulent gene was present in the isolate with a higher virulence score.

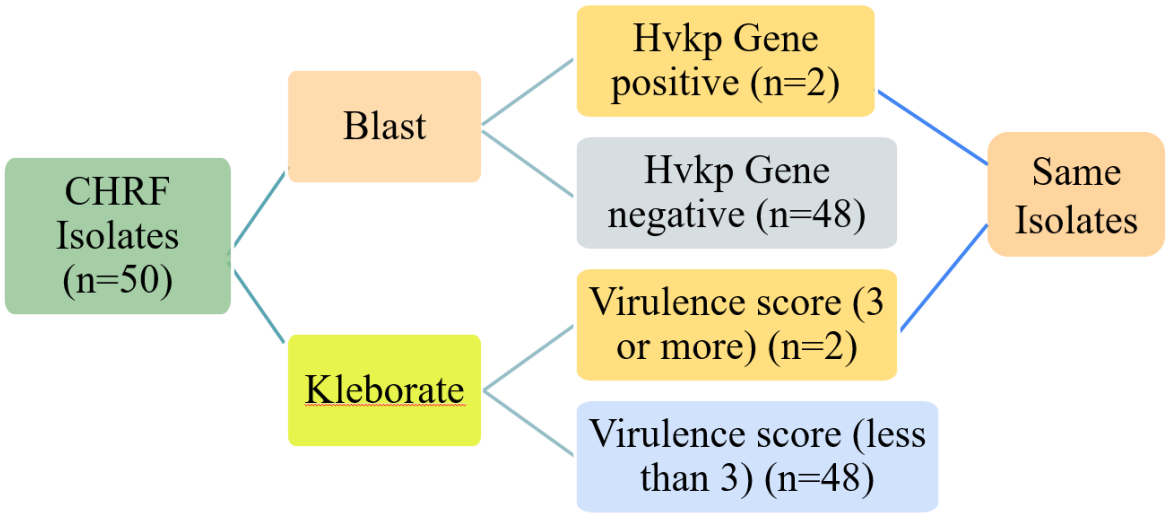

Figure-8: HvKp strain identification from CHRF samples through BLAST and Kleborate.

2.3. String Test

The string test was performed on 19 out of 50 samples, 17 of which were chosen at random using a Python random package, and two positive samples were included.

```
KPN=pd.read_csv(io.StringIO(data['list.csv']. decode('utf-8')))

randomized_sample=KPN.sample(n=20)

print (randomized_sample)
```

Figure-9: Randomizing tool of Python for obtaining 18 random sample.

After randomization, the samples were taken from the Child Health Research Foundation (CHRF) biobank, streaked in MacConkey Agar, and incubated overnight at 37ºC. MacConkey agar is a bacterial growth medium that is both selective and differential. It is designed to selectively isolate and distinguish gram-negative and enteric bacteria using lactose fermentation. In this medium, crystal violet and bile salts inhibit gram-positive bacteria. Lactose fermentation by enteric bacteria is identified using the pH indicator neutral red. Lactose fermenters change the color of the medium to red or pink, while non-fermenters do not [27]. 51.5g of MacConkey Agar powder (Peptone 20.0; lactose 10.0; Bile Salt 1.5; Sodium chloride 5.0; Neutral red 0.03; Crystal Violet 0.001; Agar 15.0) was suspended in 1 liter of distilled water to create the MacConkey agar. The mixture was autoclaved at 121ºC for 15 minutes once the powder had completely dissolved in the boiling water.

Figure-10: String Test on hypervirulent *Klebsiella pneumoniae (*>5mm).

Following *Klebsiella pneumoniae* growth in MacConkey agar, an inoculating loop was used for the string test. The loop was touched on the colony and stretched from the media to determine the string length. The colonies of different samples had variable lengths of strings based on their hypermoucoidity, and some of them did not have any hypermucoidity. The string test was considered positive only when the string length exceeded 5mm.

2.4. Understanding the clinical manifestation of patients

Finally, a comparison was made between the patients' clinical manifestations and the dry and wet lab results. To determine the relationship between the patient's clinical result and the hypervirulent *Klebsiella pneumoniae* infection, specific criteria were compared. The samples were examined for signs of disease to determine whether any of the patients had endophthalmitis, meningitis, pneumonia, necrotizing fasciitis, or non-hepatic abscesses. Additionally, in order to confirm that the suspected sample was hypervirulent *Klebsiella pneumoniae*, the antibiotic-resistance were also compared. In addition, obtaining sufficient source control and active antibiotic therapy is crucial for the management of hvKp infection. Consequently, in order to manage the infection site and comprehend the patterns of local antibiotic resistance, empirical therapeutic therapy is needed. The duration of the treatment should vary between two and six weeks based on the site and extent of infection. The empiric treatment options were compared with the ones obtained from the patient's clinical manifestation. β-lactam/β-lactamase inhibitors, fluoroquinolones, carbapenems, or aminoglycosides were used in the treatment of pneumonia, liver abscess, or other intra-abdominal infections. Third-generation cephalosporins or carbapenems were also used to treat infections of the central nervous system, and intravitreal antibiotics (cefazolin, ceftazidime, aminoglycosides, or imipenem) combined with intravenous antibiotics (variable, but typically cephalosporins) were administered to treat endophthalmitis [28]. Additionally, to comprehend the antibiotic resistance of hypervirulent *Klebsiella pneumoniae*, the antibiotic susceptibility test was compared with another article in which the susceptibility breakpoints were interpreted using the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) (2020) [29].

CHAPTER 3
RESULT

3. Result

3.1. Bioinformatics

Initially, the identification method of Hypervirulent *Klebsiella pneumoniae* was verified using BLAST and Kleborate. After the verification, the CHRF samples were obtained in order to identify the potential hypervirulent *Klebsiella pneumoniae*. Of the 50 samples, two were found to be hypervirulent *Klebsiella pneumoniae*, one possessing the virulence gene *iroBCDN, iutA, iucABCD* and scoring 5 on the virulence score and another had virulence gene *iroBCDN, iutA, iucABCD, rmpACD, rmpA2, peg-344* which includes hypermucoid gene as well and a virulence score of 4. The virulence genes and scores that have been found are indicated in Table 4.

Sample No.	**Organism**	**Virulence Score (Kleborate)**	**Hypervirulent genes (BLAST)**
1	*K. pneumoniae*	0	no virulent genes identified
2	*K. pneumoniae*	1	no virulent genes identified
3	*K. pneumoniae*	1	no virulent genes identified
4	*K. pneumoniae*	1	no virulent genes identified
5	*K. pneumoniae*	1	no virulent genes identified
6	*K. pneumoniae*	1	no virulent genes identified
7	*K. pneumoniae*	0	no virulent genes identified
8	*K. pneumoniae*	1	no virulent genes identified
9	*K. pneumoniae*	1	no virulent genes identified
10	*K. pneumoniae*	1	no virulent genes identified
11	*K. pneumoniae*	1	no virulent genes identified
12	*K. pneumoniae*	0	no virulent genes identified
13	*K. pneumoniae*	0	no virulent genes identified
14	*K. pneumoniae*	0	no virulent genes identified
15	*K. pneumoniae*	0	no virulent genes identified
16	*K. pneumoniae*	0	no virulent genes identified
17	*K. pneumoniae*	0	no virulent genes identified

18	*K. pneumoniae*	1	no virulent genes identified
19	*K. pneumoniae*	5	*iroBCDN, iutA, iucABCD*
20	*K. pneumoniae*	4	*iroBCDN, iutA, iucABCD, rmpACD, rmpA2, peg-344,*
21	*K. pneumoniae*	0	no virulent genes identified
22	*K. pneumoniae*	0	no virulent genes identified
23	*K. pneumoniae*	0	no virulent genes identified
24	*K. pneumoniae*	0	no virulent genes identified
25	*K. pneumoniae*	1	no virulent genes identified
26	*K. pneumoniae*	1	no virulent genes identified
27	*K. pneumoniae*	0	no virulent genes identified
28	*K. pneumoniae*	0	no virulent genes identified
29	*K. pneumoniae*	0	no virulent genes identified
30	*K. pneumoniae*	1	no virulent genes identified
31	*K. pneumoniae*	0	no virulent genes identified
32	*K. pneumoniae*	0	no virulent genes identified
33	*K. pneumoniae*	0	no virulent genes identified
34	*K. pneumoniae*	0	no virulent genes identified
35	*K. pneumoniae*	0	no virulent genes identified
36	*K. pneumoniae*	1	no virulent genes identified
37	*K. pneumoniae*	1	no virulent genes identified
38	*K. pneumoniae*	0	no virulent genes identified
39	*K. pneumoniae*	0	no virulent genes identified
40	*K. pneumoniae*	0	no virulent genes identified
41	*K. pneumoniae*	0	no virulent genes identified
42	*K. pneumoniae*	1	no virulent genes identified
43	*K. pneumoniae*	1	no virulent genes identified
44	*K. pneumoniae*	1	no virulent genes identified
45	*K. pneumoniae*	1	no virulent genes identified
46	*K. pneumoniae*	0	no virulent genes identified

47	*K. pneumoniae*	1	no virulent genes identified
48	*K. pneumoniae*	1	no virulent genes identified
49	*K. pneumoniae*	0	no virulent genes identified
50	*K. pneumoniae*	1	no virulent genes identified

Table- 4: Bioinformatic result by BLAST and Kleborate.

3.2. String Test

The string test was performed on 19 samples out of 50, including two samples that was positive for hypervirulent genes, in accordance with the bioinformatic findings. The samples were taken from the CHRF biobank, sub-cultured on MacConkey media, and then incubated at 37°C for 24 hours or overnight [22]. After 24 hours out of 19 samples, the string test was positive for 3 samples. Table 5 displays the string test findings for *Klebsiella pneumoniae* samples.

Sample No.	**Subculture Date**	**String Length (mm)**	**Mucoid (Y/N)**
1	03-Jun	<5mm	Y
2	03-Jun	<5mm	Y
3	03-Jun	<5mm	Y
5	03-Jun	<5mm	Y
6	03-Jun	<5mm	Y
7	03-Jun	<5mm	Y
8	03-Jun	<5mm	N
9	03-Jun	<5mm	Y
10	03-Jun	<5mm	Y
11	03-Jun	No growth	
12	11-Jun	<5mm	Y
13	03-Jun	<5mm	Y
14	11-Jun	<5mm	Y
15	11-Jun	<5mm	Y
16	03-Jun	No growth	
17	03-Jun	15 mm (approx.)	Y

18	11-Jun	<5mm	N
19	11-Jun	18 mm (approx.)	Y
20	03-Jun	20 mm (approx.)	Y

Table- 5: String Test Result.

3.3. Clinical Manifestation of Patients

In order to determine whether any of the patients had hypervirulent *Klebsiella pneumoniae infections*, the bioinformatics data and string test results were compared to the patient histories. The retrieved data were from 2009 to 2018. The sample source was blood and identification was done through the culture method. The sample site included Bangladesh Shishu Hospital and Institute (BSHI), Shishu Shastho Foundation (SSF), and Chattogram Ma-O-Shishu Hospital (CMOSH). The identification of endophthalmitis, meningitis, pneumoniae, necrotizing fasciitis, and non-hepatic abscesses was determined by analyzing case definition and the final diagnosis. It was discovered that 4 patients had meningitis diagnoses among which 1 patient died, and 3 patients had pneumoniae and severe pneumoniae diagnoses. These cases were chosen as a possibility for the Hypervirulent *Klebsiella pneumoniae* diagnosis. However, the rest of the infections and diagnoses were unrelated to the Hypervirulent Klebsiella pneumoniae infection. Table-6 states the clinical manifestations of the 50 patients.

Serial no	Organism	Method	Source (specimen type)	Study site	Hospital Admission Year	Age in month	Sex	Case definition	Outcome	Final Diagnosis
1	*K. pneumoniae*	Culture	Blood	DSH	2009	0	Male	Very severe disease	Discharged	Neonatal jaundice, Septicemia/sepsis, Meningitis, Neonatal sepsis
2	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Meningitis	Discharged	Neonatal sepsis
3	*K. pneumoniae*	Culture	Blood	CMOSH	2009	3	Male	Severe Pneumonia	DORB	Septicemia/sepsis
4	*K. pneumoniae*	Culture	Blood	DSH	2010	0	Male	Missing	Died	Pre-term low birth weight (PTLBW), Neonatal jaundice
5	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Female	Severe Pneumonia	DORB	Neonatal sepsis

6	*K. pneumoniae*	Culture	Blood	DSH	2013	0	Male	Missing	Died	Pre-term low birth weight (PTLBW), Septicemia/sepsis
7	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Meningitis	Discharged	Neonatal sepsis
8	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Very severe disease	Discharged	Neonatal sepsis
9	*K. pneumoniae*	Culture	Blood	SSF	2009	12	Male	Meningitis	Discharged	Febrile convulsion/Atypical febrile convulsion, Meningitis
10	*K. pneumoniae*	Culture	Blood	DSH	2013	0	Male	Very severe disease	Died	Neonatal sepsis, Neonatal jaundice
11	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Severe Pneumonia	Discharged	Neonatal sepsis

12	*K. pneumoniae*	Culture	Blood	CMOSH	2011	7	Male	Pneumonia	Died	Septicemia/sepsis, PEM/2° PEM
13	*K. pneumoniae*	Culture	Blood	CMOSH	2011	0	Female	Severe Pneumonia	DORB	Neonatal sepsis, Pre-term low birth weight (PTLBW)
14	*K. pneumoniae*	Culture	Blood	DSH	2016	64	Female	Not eligible	Died	Septicemia/sepsis
15	*K. pneumoniae*	Culture	Blood	DSH	2011	0	Male	Very severe disease	Died	Septicemia/sepsis
16	*K. pneumoniae*	Culture	Blood	DSH	2018	0	Female	Meningitis	Discharged	Severe perinatal asphyxia/perinatal asphyxia, HIE
17	*K. pneumoniae* (string test positive)	Culture	Blood	DSH	2011	0	Female	Very severe disease	Discharged	Neonatal sepsis, Neonatal jaundice
18	*K. pneumoniae*	Culture	Blood	DSH	2010	0	Male	Missing	Discharged	Septicemia/sepsis

19	*K. pneumoniae* (virulence score= 5)	Culture	Blood	DSH	2018	2	Male	Meningitis	Died	Acute gastroenteritis (AGE)/Acute watery diarrhoea (AWD), Septicemia/sepsis
20	*K. pneumoniae* (virulence score= 4)	Culture	Blood	CMOSH	2013	0	Male	Very severe disease	Discharged	Neonatal sepsis
21	*K. pneumoniae*	Culture	Blood	DSH	2014	0	Male	Very severe disease	Discharged	Neonatal sepsis
22	*K. pneumoniae*	Culture	Blood	DSH	2009	0	Male	Very severe disease	DORB	Neonatal sepsis
23	*K. pneumoniae*	Culture	Blood	DSH	2014	0	Female	Very severe disease	Discharged	Severe perinatal asphyxia/perinatal asphyxia, HIE, Neonatal sepsis
24	*K. pneumoniae*	Culture	Blood	DSH	2017	0	Female	Not eligible	DORB	Other congenital heart disease (PDA/ASD),

										Ventricular septal defect (VSD)
25	*K. pneumoniae*	Culture	Blood	DSH	2017	48	Male	Not eligible	Discharged	Encephalitis, Acute stroke syndrome
26	*K. pneumoniae*	Culture	Blood	DSH	2017	96	Female	Not eligible	Discharged	Chronic kidney disease/ CKD
27	*K. pneumoniae*	Culture	Blood	DSH	2012	0	Male	Very severe disease	Discharged	Severe perinatal asphyxia/perinatal asphyxia, HIE, Neonatal jaundice. Septicemia/sepsis
28	*K. pneumoniae*	Culture	Blood	DSH	2012	2	Female	Severe Pneumonia	Discharged	Ventricular septal defect (VSD), Other congenital heart disease (PDA/ASD), Pneumonia/bronchopneumonia

29	*K. pneumoniae*	Culture	Blood	DSH	2018	0	Male	Not eligible	Discharged	Neonatal sepsis, Neonatal jaundice, Pre-term low birth weight (PTLBW)
30	*K. pneumoniae*	Culture	Blood	DSH	2018	0	Female	Meningitis	Discharged	Severe perinatal asphyxia/perinatal asphyxia, HIE, Neonatal sepsis
31	*K. pneumoniae*	Culture	Blood	DSH	2018	4	Male	Severe Pneumonia	Discharged	Pneumonia/bronchopneumonia, Septicemia/sepsis
32	*K. pneumoniae*	Culture	Blood	DSH	2013	0	Female	Very severe disease	Discharged	Intrauterine Growth retardation (IUGR), Severe perinatal asphyxia/perinatal asphyxia
33	*K. pneumoniae*	Culture	Blood	SSF	2014	0	Female	Not eligible	Discharged	Neonatal sepsis, Low Birth weight (LBW)

34	*K. pneumoniae*	Culture	Blood	SSF	2009	0	Female	Very severe disease	Discharged	Septicemia/sepsis, Pre-term low birth weight (PTLBW), Neonatal sepsis
35	*K. pneumoniae*	Culture	Blood	CMOSH	2014	0	Male	Very severe disease	DORB	Severe perinatal asphyxia/perinatal asphyxia
36	*K. pneumoniae*	Culture	Blood	CMOSH	2010	2	Female	Severe Pneumonia	Discharged	Severe pneumonia
37	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Severe Pneumonia	Discharged	Neonatal sepsis
38	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Female	Very severe disease	Discharged	Neonatal sepsis
39	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Meningitis	Discharged	Neonatal sepsis, Meningitis
40	*K. pneumoniae*	Culture	Blood	CMOSH	2010	2	Male	Meningitis	Died	Meningitis

41	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Very severe disease	Discharged	Neonatal sepsis
42	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Female	Very severe disease	Discharged	Umbilical sepsis
43	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Female	Meningitis	Discharged	Neonatal sepsis
44	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Female	Very severe disease	Discharged	Neonatal sepsis
45	*K. pneumoniae*	Culture	Blood	CMOSH	2010	0	Male	Severe Pneumonia	Discharged	Neonatal sepsis
46	*K. pneumoniae*	Culture	Blood	CMOSH	2011	1	Female	Severe Pneumonia	Died	Ventricular septal defect (VSD), Septicemia/sepsis
47	*K. pneumoniae*	Culture	Blood	CMOSH	2011	0	Male	Very severe disease	Died	Pre-term low birth weight (PTLBW), Neonatal sepsis

48	*K. pneumoniae*	Culture	Blood	CMOSH	2012	0	Male	Severe Pneumonia	Discharged	Neonatal sepsis
49	*K. pneumoniae*	Culture	Blood	CMOSH	2013	0	Male	Very severe disease	Discharged	Severe perinatal asphyxia/perinatal asphyxia
50	*K. pneumoniae*	Culture	Blood	CMOSH	2013	0	Male	Severe Pneumonia	DORB	Neonatal sepsis, Intrauterine Growth retardation (IUGR)

Table- 6: Clinical Manifestation of the Patients.

3.4. Antibiotic Susceptibility Test Data

To comprehend the hypervirulent *Klebsiella pneumoniae* resistance, the patients' antibiotic susceptibility test results were obtained from the CHRF database. To determine whether any of the antibiotics were effective in treating patients with hypervirulent *Klebsiella pneumoniae,* the most often used antibiotics were considered. A variety of antibiotics, including ciprofloxacin, amikacin, gentamicin, netilmicin, imipenem, meropenem, ampicillin, cloxacillin, oxacillin, cefotaxime, ceftazidime, ceftriaxone, and cefixime, were evaluated on Mueller-Hinton agar to determine the susceptibility of *Klebsiella pneumoniae.* According to the AST, more than 41% of the isolates were resistant to 13 antibiotics. In addition, only a limited number of antibiotics, including amikacin, gentamicin, netilmicin, imipenem, and meropenem, were effective in treating *Klebsiella pneumoniae.*

Antibiotics	Number of patients	Susceptible	Intermediate	Resistant
Ampicillin	28	0	0	100%
Cefotaxime	17	0	0	100%
Ceftazidime	46	0	2%	98%
Ceftriaxone	46	0	4%	96%
Cefixime	22	0	0	100%
Chloramphenicol	39	56%	3%	41%
Cotrimoxazole	43	21%	0	79%
Ciprofloxacin	47	0	23%	77%
Amikacin	44	27%	11%	61%
Gentamicin	46	4%	9%	87%
Netilmicin	27	11%	15%	74%
Imipenem	32	22%	12%	66%
Meropenem	27	26%	11%	63%

Table- 7: Antibiotic Susceptibility Test Data in Percentage.

3.5. Comparison of bioinformatics findings with laboratory findings

A comparison of the bioinformatics data, clinical manifestation, and antibiotic susceptibility test results for the three isolates that tested positive for the string test is shown in Table 8. The data shows that sample no. 17 did not yield any virulence genes, but it produced a string length of around 15 mm, implying the possibility of virulence genes present in the sample but not certainly in the database. Additionally, sample number 19 aligns with every requirement by having virulence genes and a string length greater than 5 mm. Furthermore, the case definition reports that the patient had meningitis after developing sepsis, which ultimately led to the patient's death. This is also indicative of an infection caused by hypervirulent Klebsiella pneumoniae. Similarly, sample no. 20 contains a virulence gene with a string length of approximately 20 mm, resulting in neonatal sepsis. All the isolates were resistant to multiple antibiotics, resulting in limited treatment options.

Sample No.	Bioinformatics			String test		Clinical Manifestation			Antibiotic Susceptibility Test
	Sequence Type (ST)	**Virulence Score**	**Hypervirulent genes**	**String Length**	**Mucoid**	**Case Definition**	**Outcome**	**Final Diagnosis**	**Resistant**
17	ST411	0	no virulent genes identified	15 mm (approx.)	Y	Very severe disease	Discharged	• Neonatal sepsis • Neonatal jaundice	• Ampicillin • Ceftazidime • Ceftriaxone • Ciprofloxacin • Chloramphenicol • Amikacin • Gentamicin
19	ST268-1LV	5	• iroBCDN • iutA • iucABCD	18 mm (approx.)	Y	Meningitis	Died	• Acute gastroenteritis (AGE)/ Acute watery diarrhoea (AWD)	• Ampicillin • Ceftazidime • Ceftriaxone • Cefixime • Ciprofloxacin

								• Septicemia/sepsis	
20	ST29	4	• iroBCDN • iutA • iucABCD • rmpACD • rmpA2 • peg-344	20 mm (approx.)	Y	Very severe disease	Discharged	• Neonatal sepsis	• Ampicillin • Ceftazidime • Ceftriaxone • Chloramphenicol • Amikacin • Gentamicin • Netilmicin • Imipenem

Table-8: Comparison of Bioinformatics and Experimental lab findings.

CHAPTER 4
DISCUSSION

4. Discussion

The purpose of this retrospective study was to determine whether children of Bangladesh were infected with hypervirulent *Klebsiella pneumoniae*. Even though different researchers characterize hypervirulent *Klebsiella pneumoniae* differently, this study may provide some insight. Previously, a study on the genomic characterization of the isolates from patient samples at the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b) identified 19% hvKp from the genomes of 32 *Klebsiella pneumoniae* isolates including these sequence types: two ST 420: K20, two ST65: K2, one ST268: K20 and one ST4321: K39 [11]. This investigation was conducted in order to identify and gather additional information regarding the high virulence score hvKp that has been reported to exist in Bangladesh.

In this study, the hypermucoidity of the three samples was detected by the string test and it was found that there was the presence of virulence genes and high virulence scores. It indicates that the two strains were verified as hypervirulent since they match all the criteria to be a hypervirulent strain. Moreover, the strain with only a string test positive but no presence of virulence genes or score shows that there is a possibility of the presence of virulence gene which is not included in the database. because it may be hypothesized that the virulence gene sequences of different strains of *Klebsiella pneumoniae* differ, which renders the curated database unable to comprehend all strain sequences. The three reported hypervirulent *Klebsiella pneumoniae* isolates had sequence types ST411, ST268-1LV, and ST29. The identified sequences ST411, ST29, and ST268-1LV have not been reported in Bangladesh before, although sequence ST268 was reported previously by the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b). The detected sequence type ST268-1LV differs from the previously reported ST268 by a single allele at one of the loci. Overall, this is the first study to report these new sequence types, to the best of what is currently known. ST268 with ESBL genes was found in northern Japan and was predominant among hypervirulent *Klebsiella pneumonaie* (hvKp) [30].In addition the ST29 sequence was identified in Pakistan in abattoir wastewater. Moreover, a study conducted in Saudi Arabia found that ST29 was the most predominant ST of carbapenem-resistant *Klebsiella pneumonaie* in Riyad. Furthermore, an ESBL *Klebsiella pneumonaie* generating ST29 was found in both local and imported poultry meat in Ghana [31]. ST29 was also identified in the United States among 5 bloodstream isolates [32]. In China, an isolate of NDM-5 carbapenemase-producing hypermucoviscosus *Klebsiella pneumonaie* was found in a sputum sample belonging to ST29 [17].

Additionally, a retrospective investigation conducted in China in 2014 identified ST29 as K54 serotype ESBL-producing *Klebsiella pneumoniae* [33]. However, ST411 was not reported anywhere, either locally or globally. To accommodate more virulence genes in the database for future implementation, Prokka can be used to identify the virulence genes of further strains. Moreover, it was discovered that all 3 patients were diagnosed with sepsis or neonatal sepsis following a case definition of meningitis. Additionally, clinical manifestations revealed that a total of four patients were diagnosed with meningitis (one fatal case), and three with pneumonia or severe pneumonia. According to the AST, more than 41% of the isolates were multi-drug resistant and the limited number of antibiotics were effective in treating *Klebsiella pneumoniae.* The isolates with positive string test and a high virulence score also exhibited multi-drug resistance. Thus, this raises the possibility of difficulty in treating hypervirulent *Klebsiella pneumoniae* in the future. Design of PCR tests for quick identification for hypervirulent strains can be useful. When PCR results are reported ahead of time, clinicians have a greater ability to execute informed decision-making and can treat patients with appropriate antibiotics. Finally, developing an effective vaccine against HvKp strains and identifying the antibiotic-resistant gene can help prevent HvKp infection among children.

5. Limitations

The NCBI isolates could not be thoroughly analyzed despite the data verification since the samples could not be examined in the experimental lab. Furthermore, 19 samples were obtained for the string test, and only 50 samples were taken into consideration for the purpose of this study. To comprehend the hypervirulence of *Klebsiella pneumoniae,* a larger sample pool is needed. Furthermore, strain-to-strain variations in hypervirulent gene sequences suggest that the database of virulence genes is not yet comprehensive. To fully understand both the presence of hvkp and its gene expression, additional *Klebsiella pneumoniae* characterization, and sequencing are necessary. Since the virulence gene sequence varies, a repository for hvKp serotypes should be developed, and the database should be updated frequently. Additionally, more genes must be identified using bioinformatics tools like Prokka in order to comprehend the prevalence and presence of virulence genes in *Klebsiella pneumoniae.* To fully understand infections and predict severity, additional patient information and a history of antibiotic resistance are also required.

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