Synopsis of the thesis on

Genome based analysis and phenotypic study of antibiotic resistance and virulence factors in *Klebsiella pneumoniae*

Synopsis for a Ph.D. thesis submitted by:

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1 Introduction:

1.1 *Klebsiella pneumoniae*: *Klebsiella pneumoniae* (Kp), a member of the Enterobacteriaceae family, is one of the commensal organisms in the gastrointestinal tract of healthy humans and animals (1). Kp was also declared as a "Priority 1: CRITICAL" pathogen by the World Health Organization (WHO) in 2017 (2), while it was listed in the Indian priority pathogen list (IPPL) in 2021 (3), due to the increasing antibiotic resistance (ABR), including last-resort antibiotics such as carbapenems, colistin, and tigecycline.

1.2 Epidemiology: The global presence of carbapenem resistance in Kp is mainly due to the presence of isolates containing class A-type *b*-lactamase (*bla*KPC), class B-type metallo-*b*- lactamase (*bla*NDM), and class D-type oxacillinase (*bla*OXA-48), with geographical variation. For example, Greece, Taiwan, Columbia, the USA, Canada, and China have many more strains that produce *bla*KPC and *bla*NDM than strains that produce *bla*OXA-48, which are far less common in those nations. In the Arabian Peninsula and India, *bla*OXA-48 and *bla*NDM producers are common while *bla*KPC producers are rare.

The pathogenicity of *K. pneumoniae* bacterium is linked to a number of virulence characteristics that allow it to avoid detection by the host's innate defense responses. Capsules, exopolysaccharides associated with mucoviscosity, lipopolysaccharides (LPSs), adhesins, and iron absorption systems are all *Klebsiella pneumoniae* virulence factors. Multiple drug resistance and the capacity of *K. pneumoniae* to induce nosocomial infections in people are variables that aggravate the infection caused by *K. pneumoniae*. Furthermore, the earlier occurrence had six Asian patients presented to a

US hospital with *K. pneumoniae* liver abscess; the gastrointestinal system was suspected as a route of entry in one of the cases (4).

1.3 Antimicrobial resistance in *K. pneumoniae:* The resistance rate against carbapenems and extended-spectrum *b*-lactamases (ESBLs) has been worryingly increasing in India in the past few years. A report from India has manifested an increase in carbapenem resistance rates from 9% in 2008 to 44% in 2010 (5). Another report from a tertiary hospital in India reported 24.6% resistance to ESBLs in 2007 (6). In 2017, carbapenem resistance was reported as high as 44% in India (7). In 2018, a significant rise in the resistance level of ESBLs (45.1–93.1%) was reported in India, and the highest resistance of 84.9% was reported against cephalosporins (8). A report from North India suggested a 29.4% resistance rate against carbapenems (9). Recently, the overall prevalence of multidrug resistance [MDR: isolate that has developed resistance to at least one antimicrobial agent from three or more antimicrobial categories (10)] among Indian Kp isolates was reported at 58.0%. Further, it was also reported that tigecycline and colistin were the most effective drugs so far. However, extensively drug-resistant (XDR: resistant to at least one agent in all antimicrobial groups except two or fewer, i.e., bacterial isolates remain susceptible to only one or two categories) isolates with co-resistance against carbapenem and colistin (7) and pan drug-resistant (PDR: resistant to any antimicrobial agent) isolates have also recently been reported in India.

1.4 Virulence factors in *K. pneumoniae:* *K. pneumoniae* uses a variety of tactics to proliferate and defend itself from the host immune response. To date, four key groups of virulence factors have been thoroughly characterised in *K. pneumoniae,* these virulence factors are capsule, lipopolysaccharide (LPS), siderophores, and fimbriae, commonly

known as pili (11). Several other factors have been recently identified as being important for *K. pneumoniae* virulence. However, these factors are not yet thoroughly characterized, and much work remains to be done to fully understand their mechanisms of action and clinical significance. These virulence factors include OMPs, porins, efflux pumps, iron transport systems, and genes involved in allantoin metabolism. Among the above-mentioned, the capsule is a crucial acridity factor that is implicated in at least two pathogenic pathways, namely the protection of bacteria from phagocytosis and direct suppression of the susceptible host response. Several capsule types (K), including K1, K2, K54, K57, K20, and K5, are frequently related to the pattern of community-acquired invasive pyogenic liver abscesses, septicemia, and pneumonia. Furthermore, K1, K2, K20, K54, and K57 are primarily harmful to experimental infections in mice and are usually connected with severe infections in humans (12).

2. Rationale:

K. pneumoniae has become an increasing concern in the clinical environment due to the rise in the number of lethal infections. Due to the increasing number of multidrug-resistant, especially carbapenem-resistant and hypervirulent pathotypes along with antibiotic resistance among strains, which can infect both immune-compromised and healthy individuals have become critical in prevention and control.

Treatment of *K. pneumoniae* infections has become a major problem in the medical field due to the high rate of capsule production, colonization with the help of fimbriae and antibiotic resistance. Also, due to the increasing antibiotic resistance including last-resort antibiotics such as carbapenems, colistin, and tigecycline. Further, carbapenem-resistant *Enterobacteriaceae* (CRE) are emerging at an alarming rate; hence, surveillance studies

of MDR *K. pneumoniae* have become highly important.

Genotypic characterization will help to understand the prevalence of antimicrobial resistance and virulence genes among ST types, also novel mutations and SNPs.

3. Objectives:

1. Sample collection, identification, screening of antibiotic resistant (ABR) isolates, and whole genome sequencing.
2. Genotypic and phenotypic study of antibiotic resistance in *K. pneumoniae*.
3. Genotypic and phenotypic study of virulence factors in *K. pneumoniae*

4. Results:

Work done for,

4.1 Objective 1: Sample collection, Identification, Screening of Antimicrobial resistanct isolates and whole genome sequencing

Collection, isolation, and identification of clinical isolates (*n* =30) of Urinary Tract Infections (UTIs), Blood Stream Infections (BSIs), and Respiratory Tract Infections (RTIs) *Klebsiella pneumoniae.* All samples were collected from Metropolis and Sterling Lab, Vadodara, Gujarat, India. The following isolates were grown on MacConkey agar plates. Primary detection and antimicrobial susceptibility testing of isolates were done by the Vitek 2 system, Minimum inhibitory concentrations of Colistin and Tigecycline were done according to CLSI's Micro broth dilution method. Further Isolates were confirmed as *K. pneumoniae* by 16 sRNA sequencing and NCBI *Bla*st, all isolates (*n*=30) were found as *K. pneumoniae*. Then all isolates were subjected to whole genome sequencing using Ion Torrent and Illumina Miseq sequencing platform at Gujarat Biotechnology Research Centre, Gandhinagar, Gujarat, India. Further, all *K. pneumonioae* isolates (*n*=30) were classified as MDR, XDR, PDR, and Susceptible based on their resistance pattern against different classes of drugs according to Magiorakos et al., 2012 (10).

4.2 Objective 2: Genotypic and phenotypic study of antibiotic resistance in *K. pneumoniae*

The quality of Raw data of all WGS isolates was checked, and trimming was done for a few isolates, *de novo* assembly was prepared followed by quality assessment of genome assembly, Largest contig, N50, GC%, and the number of contigs were listed.

Further to understand the diversity statistics of beta-lactamases genes, STs, and plasmids among Pan-India *K. pneumoniae* genomes, an additional *n*=187 genomes of *K. pneumoniae* submitted to public databases from India were retrieved.

In Multi-locus sequence typing (MLST) analysis among Pan-India genomes including our genomes (*n*=22), ST231 was found most predominant followed by ST147, ST2096, and ST14. Two novel sequence types ST5438 and ST5439 were also identified in lab isolates. In antibiotic resistance gene analysis majority of isolates were found to be producing ESBLs genes (*bla*CTM-15, and *bla*TEM-1), Carbapenemase genes (*bla*OXA-232, and *bla*NDM-5), and another beta-lactamase (*bla*TEM-1, *bla*SHV-1, and *bla*SHV-11). In correlation of carbapenemases with STs, *bla*OXA-232 was mainly detected in ST231, ST2096, and ST14. *bla*NDM-5 was majorly detected in ST147, and ST395. While ESBLs such as *bla*CTX-M-15 was mainly associated with ST231, ST147, ST2096, ST14, ST16, ST43, ST395, and ST11; and *bla*OXA-1 was detected in ST2096, ST14, and ST147.

In other than Beta-lactamases, some other antibiotic resistance genes were also detected in lab isolates, based on the analysis, *FosA* & *oqxA* gene was detected in all isolates, whereas *aac(6')-lb-cr*, *aadA2*, *mph*, and *erm(B)* were mostly associated with XDR and PDR. *sul* and *dfrA* were present in drug-resistant isolates. Further mutations in mgrB, PhoPQ, and PmrAB sequences were also checked to correlate the colistin resistance in isolates M47, M48, and M50, followed by mutation analysis in ramR sequences for tigecycline. All tigecycline resistant isolates had at least one mutation in its amino acid sequence of ramR.

In Plasmid analysis Col and Inc, two types of plasmids were detected and distributed among isolates. In correlation of plasmids with STs, for instance, most ST231 genomes carried Col440I and ColKP3, while few genomes of ST231 carried

ColRNAI. ColRNAI was mainly associated with ST2096 and ST23. ST14 and ST2096 mainly carried ColKP3. Col(BS512) was abundantly present in ST147 genomes, while not frequently carried by ST231, ST14, and ST2096. ColpVC was seen to be associated mainly with ST43 and little with ST147 and ST437. Among prevalent STs, Col440II was carried by ST16, ST23, and ST101. Regarding IncF plasmids, different sets of IncF plasmids were observed to be associated with different STs. IncFIA, IncFIB(pQil), IncFII(K), and IncF(pAMA1167-NDM-5) were abundantly present in ST231 genomes, whereas ST14 harbored only IncFII(K) and IncFIB(K), which were not carried by ST231. A few STs: ST395, ST147, ST43, and ST11, were found to be associated with IncFIB(pQil). IncHI1B(pNDM-MAR) was carried mainly by ST2096, ST14, and ST43. IncFII was carried by the genomes of ST147, ST395, ST14, and ST16. IncFIB(pNDM-Mar) was detected mainly in ST2096 and ST14. IncR was observed to be strongly associated with ST147, while IncFIB(pKPHS1), IncFII(pKPX1), and ColpVC were also detected in the genomes of ST147.

In the subsequent study, we chose a few isolates based on the presence of the carbapenemase gene: no carbapenemase (M40), single carbapenemase producers (*bla*OXA-232 [M52], *bla*OXA-181 [J20], *bla*NDM-1 [M39], and *bla*NDM-5 [M53]), and dual carbapenemase producers (*bla*NDM-5+*bla*OXA-181 [M49], and *bla*NDM-1+*bla*OXA-232 [M17B]). Genome analysis reveals the either loss of porins or mutation while efflux-related genes were also fetched. there were no significant differences among isolates were observed. Mutation p.A217S in ompK36, mutation p.I70M, and p.I128M in ompK37 were the most common among isolates. Whereas *KpnG/H* and *marA* were the most common efflux present in genomes. Its phenotypic resistance against two carbapenem drugs (ertapenem and meropenem) was checked

and we found NDM-5 followed by OXA-181 and NDM-5+OXA-181 carrying isolates showed high MIC values. Further, no significant differences were observed either in the presence of efflux pumps or mutations in porins among isolates. By molecular docking, among single amino acid differences between OXA-181 and OXA-232 and with two amino acid differences between NDM-1 and NDM-5, OXA-232 and NDM-5 showed a higher binding affinity than OXA-181 and NDM-1 with both antibiotics. It is concluded that the presence of specific carbapenemases or combinations of the same can drastically increase MIC values. The presence of NDM-5, and OXA-181, or their combinations is more fatal than NDM-1+OXA-232.

4.3 Objective 3: Genotypic and phenotypic study of virulence factors in *K. pneumoniae*

In virulence factors, K-locus and O-locus of our genomes (*n* =30) including additional genomes (*n* =321) from INDIA were determined in which K51 capsular type was predominant followed by K64, K2, K10, and K30. In O-locus typing O1 serotype was most circulating among isolates, followed by O3/O3a, and O2a. And based on the bioinformatics analysis % virulence genes were found among O-seroypes in order-O1>O2a>O3/O3a>O2afg>OL101>O3b. The presence of genes for siderophores and fimbriae was also checked in our genomes (*n* =30). The presence of *fimA, fimH, mrkA*, and *mrkD* were seen in all isolates except 3 isolates (M10, M20, and M33). Further, phenotypic assays were carried out for different virulence factors for selected isolates considered as representative.

4.3.1 Study of Capsular polysaccharide (CPS) and Lipopolysaccharides (LPS): All isolates were tested for string phenotype and all were detected string negative except isolates M20 and M58. Based on genotypic analysis and string phenotype *n*=9

isolates were selected for phenotypic study of CPS and LPS. Capsule staining was done for all selected isolates and the size of the capsule was determined by counting at least 100 cells/isolates. Based on the analysis it was found that M48 had the highest capsule size and the lowest size was detected in isolate DJ. In the mucoviscosity assay, M58 was detected as highly mucoviscous while M17B was lowest. Phagocytosis assay was also done using isolated neutrophils of 5 healthy individuals. In FACS analysis it was detected that isolates M51 and M49 had the highest susceptibility towards phagocytosis while isolates DJ and M20 had the lowest susceptibility towards phagocytosis. In the serum killing experiment, serum was isolated from the whole blood of 5 individuals, isolates were incubated in pooled serum for 0, 1, 2, and 3 hours then plated on MHA, results were obtained after overnight incubation and isolates were categorized as serum sensitive at grades of 1–2, intermediately sensitive at grades of 3–4, and resistant at grades of 5–6. To correlate O-type and K-type with serum killing data, isolates (*n*=3) with a combination of K64+O2a were either serum sensitive (*n*=1) or intermediate sensitive (*n*=2), while isolates (*n*=3) with a combination of K51+O1 were either resistant (*n*=2) or sensitive (*n*=1). Also, both string-positive isolates were showing sensitivity against serum.

4.3.2 Study of Siderophores: In qualitative estimation of isolates, all isolates were found positive for siderophore production. Isolates were grown on CAS media to check the production of siderophores by observing the zone of clearance. Quantification of siderophores was also done for all isolates, and % siderophores were detected in a range of 1-25%. Then few isolates (M10, M33, M48, M50, M51, and M52) were selected for further experiments based on their genome profile and siderophore-producing capacity. Study of growth pattern and % siderophore

production of selected isolates were also done in the presence of growth enhancers (Iron, Nickel), growth inhibitors (DIP), and also some antibiotics (Ciprofloxacin, colistin, and ampicillin) with different concentrations. Maximum growth was observed with the presence of iron, while there was no siderophore production detected in all isolates.

Variation in growth as well as % siderophore production was observed among isolates. In the presence of nickel highest siderophore was detected in isolate M10, while no siderophore production was observed in M50 and M51. Maximum growth and less siderophore production were seen to be associated with in presence of ampicillin, whereas maximum siderophore among isolates was detected in the presence of ciprofloxacin.

5. Summary:

The present study was done to understand the antimicrobial resistance pattern mainly beta-lactamases and virulence-associated genes in clinical samples of *K. pneumoniae*.

Initially, isolates were categorized as MDR, XDR, PDR, and susceptible based on their resistance pattern for antimicrobial study. Pan-India *Klebsiella* genomes were studied and found that *bla*OXA-232, *bla*NDM-5, and *bla*CTX-M-15 were potential targets for diagnostics, while ST16 and ST14 were detected as emerging treats, due to dual producers of carbapenemases. In the virulence study, K51 and K64 were detected as the most prevalent capsular type, while O1 and O3/O3a were detected as the most circulating O-serotype among isolates. There was no correlation between K-locus and phagocytosis by neutrophils observed, while isolates with a combination of K64+O2a were either serum sensitive or intermediate sensitive; with a combination of K51+O1 were either resistant or sensitive. Also, both string-positive isolates showed sensitivity against serum. Genes of type1 and type3 fimbriae were detected in all isolates except a

few. All isolates were string negative except two isolates (M20 and M58); production of siderophore was detected in all isolates. Variations in siderophore production were observed among isolates in the presence of growth promoters, inhibitors, and antibiotics. In overall conclusion, it was observed that ST231 which mostly belongs to the K51 and O1 serotype is a more dangerous sequence type, which carries OXA-48-like genes along with more virulence genes.

6. References:

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