

ABSTRACT

The significant rise in antibiotic resistance in *K. pneumoniae*, along with enhanced virulence, is now a serious concern. *K. pneumoniae* have become resistant to carbapenem medicines are included as critical pathogens in priority 1 on both the Indian priority pathogens list (2021) and the World Health Organization's priority pathogen list (2017 & 2024). The death rate in newborn children and those with weakened immune systems has grown as a result of a lack of early identification of these strains. There was also a gap in studies focused on the genetics of antibiotic resistance and virulence factors specific to India, as well as the spread of these factors. In order to comprehend the genomics of antibiotic resistance and virulence in *K. pneumoniae*, we used the whole genome sequencing method. This methodology is considered the most suitable and provides a higher level of detail in understanding the genetic makeup of these pathogens compared to other molecular or biochemical approaches.

Total 30 identical isolates were selected for whole genome sequencing after being identified by *16S rRNA* sequencing. Among selected isolates, 5 were sensitive to drugs, 6 were multidrug-resistant (MDR), 16 were extensively drug-resistant (XDR), and 3 isolates were pandrug-resistant (PDR) detected using the Vitek2 system and the microbroth dilution method. The raw sequencing data were evaluated using several bioinformatics tools, including FastQC, Spades, Quast, and Prokka, and all isolates, except for isolate M3, yielded good results. In addition, the MLST tool was used to establish the sequence types. Antibiotic resistance genes were identified using the CARD and ResFinder databases. Plasmids were recognized using the PlasmidFinder program, while mlplasmids was utilized to determine the placement of genes in the genome. The virulence genes and prophages were analyzed using the virulence gene identification tool in the pasteurMLST database and the Phaster tool, respectively. In phenotypic experiments, such as the biofilm formation test, included doing prophage induction using the microplate crystal violet assay and lower quantities of the Mitomycin C drugs. In addition, a string test was conducted on a 5% sheep blood agar medium. The size of the capsule was then measured using Maneval's staining technique, and the size was determined using ImageJ software. The experiment on neutrophil phagocytosis was also performed using a Flow cytometry system to see and measure the fluorescence. Additionally, serum killing assay was performed using pooled serum from five individuals, adhering to the spread plate approach. The presence of siderophores was estimated qualitatively and quantitatively, both in the absence and presence of iron, iron chelator (DIP), and antibiotics. Chrome Azurol S (CAS) agar dye was used for testing.

According to our research, ST231 was identified as the predominant strain circulating in India, followed by ST147. However, later on, ST14 exceeded ST147 in terms of circulation. This research specifically examined beta-lactases, which are the primary genes lead to antibiotic resistance, in the context of genomics of Antibiotics resistance. The gene *bla*OXA-232 was identified as the most widespread gene, followed by *bla*NDM-5 and *bla*OXA-181. The bulk of these genes were found on plasmids and were recognized in the genomes of the often-occurring ST231 and ST147. This research also identified a small number of dual carbapenemase producers, and they demonstrated that the MIC >512 ug/ml for carbapenem antibiotics is caused by the presence of *bla*NDM-5, *bla*OXA-181, or their combinations, and is thus more lethal than *bla*NDM-1 + *bla*OXA-232. The overall study of antibiotic resistance showed that the XDR and PDR isolates were mostly linked to two sequence types, which were named ST231 and ST147. These isolates had either the *bla*OXA-48-like or *bla*NDM carbapenemase gene and also conferred genes such as *fosA* for fosfomycin resistance; *oqXA/B*, *aac(6')-Ib-cr* for fluoroquinolones; *aac(6')-Ib-Hangzhou*; *rmtF*, *aadA1/2* for aminoglycosides; *mph*, *erm(B)* for macrolides; *tet* for tetracycline resistant; *arr-2* for rifampin; *sul2*, *dfrA* for folate pathway antagonist; and *catA1* that confer resistance to phenicols. Additionally, tigecycline and colistin resistance were investigated. There was one mutation (T246A) in the PmrB amino acid sequence of isolates resistant to colistin, while there were several mutations (M184V being the most prevalent) in the ramR amino acid sequence of isolates resistant to tigecycline detected. However, the challenges of antimicrobial resistance and biofilm-mediated challenges might potentially be solved by using the genetic material of bacteria itself, known as prophage. Prophages has shown the ability to reduce biofilm formation in the majority of cases when induced by modest doses of mitomycin C.

In the investigation of virulence, the Capsular and O-Serotype combinations K51, K64, and O1, O3/O3a were shown to be the most often occurring. These combinations were mostly linked to the ST231, ST14, and ST147 strains. In addition, the majority of common ST231 (K51 + O1) combinations exhibited the presence of numerous virulence genes, including aerobactin, which is a hallmark for hypervirulent strains. Another combination, ST23 (K1 + O1), was also identified as a potentially problematic strain in the future because it held a huge number of virulence genes in its genome. An identical isolate (M58) exhibited the string positive phenotype. To replicate the string positive isolate, an additional known string positive isolate, M20, was added for further investigation. Variations in the size of the capsule and mucoviscosity were also observed. The

combination of K51 + O1 demonstrated the highest resistance to serum and a lower susceptibility to phagocytosis by neutrophils. Specifically, O1, which is the most prevalent, accounting for approximately 60%, displayed the maximum serum resistance. Siderophore synthesis was seen in all isolates, however variations in amount were noted. In the presence of iron, all isolates showed maximum growth, but siderophore production was very low or absent. However, the growth was significantly inhibited by DIP, and a slight increase in siderophore formation was observed. Additionally, the presence of the antibiotic ciprofloxacin was found to increase siderophore production, while ampicillin surprisingly reduced production in most isolates.

In conclusion, we advocate doing further surveillance studies in India, particularly in the Western and Northern regions. Based on the existing data, we suggest monitoring ST231, ST14, and ST147 since these strains are particularly worrisome due to their possession of several antimicrobial resistance genes. The genes *bla*OXA-23 & 181, and *bla*NDM-5 & 1, in addition to *bla*CTX-M-15, may serve as diagnostic targets for the detection of antimicrobial resistance. It is important to monitor additional isolates that produce dual carbapenemase, since they may significantly increase the minimum inhibitory concentration (MIC) to above 512 ug/ml. Prophage induction therapy, an alternative treatment, has the potential to address this issue. However, more study is required to have a clearer understanding. Moreover, due to the high prevalence of the O1 serotype and the overall limited variances in O-serotypes in India, it is possible that this might be a promising candidate for vaccine development. The ST231 strain is once again causing worry because to its high virulence, as are other strains such as ST23 (K1 + O1). It is important to continuously monitor these strains in order to effectively address the challenges they provide. Additionally, in the context of alternative treatment, limiting free iron availability to *K. pneumoniae*—through iron chelation and obstruction of iron acquisition pathways—offers a viable approach for combating multidrug-resistant infections. The selection of antibiotics should always be done with caution. Otherwise, drugs like ciprofloxacin might increase siderophore synthesis in drug-resistant *K. pneumoniae* without impacting bacterial growth, so promoting the proliferation of the pathogen. However, more research with larger number of isolates is necessary to provide a clearer understanding.