Association of Sequence types, Antimicrobial Resistance and Virulence Genes in India isolates of *Klebsiella pneumoniae*: A Comparative Genomics Study

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Abstract

Klebsiella pneumoniae is an important ESKAPE pathogen that causes sepsis, urinary tract infections, peritonitis, intraabdominal abscesses and upper respiratory infections. The strains exhibiting multidrug resistance and hypervirulence are priority pathogens for which immediate treatment and dissemination prevention strategies are required. The hypervirulent drug resistant *K. pneumoniae* is associated with high mortality rates. Numbers of environmental strains also have acquired virulence genes. Hence to gain a better understanding of the spread of antimicrobial resistant genes across the country over 10 years and to delineate environmental and clinical *K. pneumoniae*, a comparative genomics investigation was made. This is the first comparative genomic study using India isolates of *K. pneumoniae*, which includes publicly available WGS of 144 clinical and 9 environmental strains collected during 2010–2020. The *blaCTX-M-15* was widely distributed in clinical isolates since 2013 and increased over time from 5 % to 30 %. The co-existence of *blaNDM* and *blaOXA* was observed in 22 % of clinical strains. Diverse serotypes were found among the 153 *K. pneumoniae* isolates, of which, K51 (28%) and K64 (21.56%) were majorly found. Most of the K51 isolates belong to ST231 (93.02 %). And more than 50% of KL51 strains were found to have both *rmpA* and *magA*. The number of associated virulence genes (*rmpA, magA, entB, ybtS, iutA, alls*,) appeared to be higher in ST231-KL51 and ST23-KL1 isolates. Of greatest concern, these virulence genes are observed in environmental strains aswell. More than 97% of clinical strains have yersinibactin (*ybtS*), aerobactin (*iutA*) genes. Importantly, 98% of ESBL and 62% of carbapenamasen isolates harboured *ybtS, iutA* and *rmpA, magA* respectively. The IncF conjugative plasmids are predominant in *K. pneumoniae*, which contribute to the spread of AMR, and virulence genes. The increasing trend in hypervirulent strains was observed from 2017. The phylogenetic analysis separates the environmental from clinical strains and is characterized by uncommon STs and serotypes. Thus, the study illustrates the *K. pneumoniae* genomic surveillance in India representing the phylogenetic evolution, STs, AMR, virulence, serotype to provide more attention for immediate treatment and preventing the dissemination of *K. pneumoniae.*

Introduction

Klebsiella pneumoniae has a strong association with human as resident flora and colonizes almost every part of the human body with most preferential in the respiratory, gastrointestinal, and urinary tract (Paczosa & Mecsas, 2016). The other predominant habitats include soil, plants, surface water, sewage, industrial effluent, etc. (Parkinson et al., 2007; Bagley, 2014). Despite the source of origin, clinical isolates causing nosocomial and community acquired infections. This leads to serious therapeutic threats given the increase in drug resistant phenotypes and decrease in effective antibiotics (Van Duin & Paterson, 2016). The emergence and spread of extensive drug resistant *K. pneumoniae* are global concern, which causes severe untreatable infections in immunocompromised patients (Bertelli et al., 2019; Lee et al., 2017). The associated character of antibiotic resistance and virulence in *K. penumoniae* was reported to have high morbidity and mortality rates in immunocompetent individuals as well (Effah et al., 2020). Pneumonia, meningitis, urinary tract infections, and bloodstream infections are the potential illness

caused by this bacterium in immunocompromised (Paczosa & Mecsas, 2016). To treat these infections, similar classes of antibiotics are been in use for the last 2 decades, that render the bacterium to evolve strategies to survive in antibiotic stress environment (Roemhild & Schulenburg, 2019). Few potential survival strategies include acquisition of new AMR genes, production of hydrolytic enzymes, expression of efflux proteins, and formation of biofilm (Uruén et al., 2021; Ni et al., 2020). The majority of AMR genes are acquired through conjugation, transformation, and transduction. Hence understanding the co-occurrence of plasmid type and AMR gene transfer could help to limit the dissemination of AMR and virulence genes. Nevertheless, antibiotic resistance is not confined to clinical strains alone, it is also widespread among the non-pathogenic environmental isolates (Peterson & Kaur, 2018). Some important virulence factors of *K. pneumoniae* include capsular polysaccharide, siderophores, fimbriae etc. (Zhu et al., 2021). However, few factors are common in both clinical and environmental isolates such as capsule, *type 1 fimbriae, type 3 fimbriae* and siderophores.

K. pneumoniae are classified into classical strain (cKp) and hypermucous strain (hvKp) (Russo & Marr, 2019). The hvKp is significantly different from the cKp by harbouring *rmpA* and *rmpA2* mucus-regulator genes, K1, K2, K20 capsular types and aerobactin (Russo & Marr, 2019). The hvKp strains can cause serious infections such as liver abscess and meningitis in both immunocompetent and immunocompromised individuals (Serban et al., 2021). Also, hvKp strains are characterized by increased resistance to host defense mechanism and are associated with high mortality rates (Shon et al., 2013). Both cKp and hvKp are known to possess high diversity of antimicrobial resistance genes, with increased resistance towards 3rd generation cephalosporin (Xie et al., 2018). The combination of drug resistance and hypervirulence advocates *K. pneumoniae* as a clinically significant pathogen of global health concern. Also, *K. pneumoniae* is listed in WHO priority critical pathogens. As the hypervirulent and drug resistant population remains to overlap in most isolates, it is necessary to identify the distinguishing features of pathotypes and environmental isolates of *K. pneumoniae* to prevent the spread of pathogenic strains in hospital and community settings.

The traditional taxonomic techniques, 16S gene Sanger sequencing, and PCR-based sequence typing (MLST) are all well-established in characterizing pathogenic bacteria (Linxiang Chen et al., 2014). However, these techniques are unable to distinguish closely related species as well as limited in detecting diverse AMR genes and virulome. Recent advancements in the next generation sequencing and extended applications of bioinformatics tools facilitate gathering information on thousands of bacterial species on their virulence genes, resistance genes, and genetic relationship (Hendriksen et al., 2019). In the present study, comparative investigations on the distinctive features of *K. pneumoniae* were studied. We retrieved 153 genomes of clinical and environmental *K. pneumoniae* isolates of India origin from PATRIC and comparative genomic analyses were made to understand the spread of *K. pneumoniae* resistant strains across the country over 10 years. Followed by, sequence types (ST), evolutionary relationships, the resistance mechanisms, acquisition of AMR genes, presence of conjugative and non-conjugative plasmids and virulence genes were screened. In addition, the association between the four major factors such as virulence, resistance determinants, replicon types and ecological niche were studied for 153 genomes of *K. pneumoniae.* The genomic surveillance is conducted to gain insights into genotypic

characterization which may facilitate immediate treatment and attention to prevent the dissemination in the environment and clinical settings.

Materials And Methods

Retrieval of 153 genome sequence data

The Whole Genome Sequence (WGS) of 153 India isolates of *K. pneumoniae* were collected from the PATRIC database (The Pathosystems Resource Integration Center) (Wattam et al., 2018). We retrieved the assembled FASTA sequence for 153 strains which were previously assembled using Spades. These strains were sequenced using Illumina, Nanopore and PacBio sequencing platform and shown in the Supp. table-1. The ST type, year of strain isolation and sampling source of the 153 isolates were collected from the PATRIC. Also, the genome size and GC content of each strain were retrieved. For further analysis FASTA format was used.

Antimicrobial Resistance profiling

AMR profiles of each isolate were carried out using, Resfinder 4.1 in the CGE database (Kwon et al., 2016). A total of eight antibiotic classes such as β-lactam, aminoglycoside, fluoroquinolone, fosfomycin, sulfonamide, macrolide, phenicol and rifamycin were selected. For AMR profiling, 88 AMR genes belonging to these classes were taken. Followed by, the resistance mechanism of 153 isolates was analyzed using CARD (Comprehensive Antibiotic Resistance Database) (McArthur et al., 2013).

Virulome analysis

VFDB (Virulence factor database) was used to screen the virulence genes. This tool allows the identification of type I fimbriae genes (*fimD, fimK, fimH, fimC)*, type III fimbriae genes (*mrkD, mrkJ, mrkF, mrkC, mrkA, mrkI*), siderophore genes (*iutA, entB, ybtS*), allantoin utilization gene (*allS*), capsular genes (*wcaH, wcaG, manB, manC, hcaA, wcbR, rmpA* and *magA*), type IV pili (*tapQ, pilJ*). type III secretion system (*vopB2*) and type IV secretion system (*clpV/tssH*) [http://www.mgc.ac.cn/VFs/] (Kwon et al., 2016).

Serotype analysis

To screen the type of serotype, Kaptive database was used (Wyres et al., 2019). Using this type K-locus types were predicted.

Plasmid profiling

Plasmid finder was utilized to identify plasmids that are found in the genome sequence. The FASTA format was used to identify the replicon sequence that matched with 100% identity (Carattoli et al., 2014).

Phylogenetic analysis

The evolutionary relationships among the 153 *K. pneumoniae* were determined using CSI Phylogeny. To investigate the phylogenetic placement based on MLST, the seven housekeeping genes were concatenated for each genome and a reference strain, MGH 78578 (Kumar et al., 2011) using MegaX. Neighbor-joining method was used to view the tree with bootstrap value 1000. Tree editing and annotation were performed using interactive Tree of Life (iTOL) (Nicolás et al., 2018).

Results

Overview of the Genomic metadata Composition of *K.pneumoniae* isolates

The PATRIC metadata depicts 144 clinical strains and 9 environmental strains. The isolation source of 153 strains are blood (n=115), urine (n=9), endotracheal aspirate (n=5) bronchoalveolar lavage (n=4), sputum (n=4), pus (n=3), CSF (n=2), nasal swab (n=2), rhizosphere soil (n=1; AWD5), root nodule (n=1; HPCN22), domestic sewage (n=2; HPCN5, HPCN17), STP sludge (n=1; PVN-1), endophyte (n=1; ME30), river surface water (n=1; PL1-RCS238), industrial waste water (n=1; EGD-HP19), agriculture field soil (n=1; KBG6.2) Supp.table 1. The genomic characterization of *K. pneumoniae* was performed as first part of the study. From the PARTIC database, we found these genomes were sequenced majorly by illumina and ion torrent. The genomes of clinical strains (n=144) and environmental strains (n=9) ranged in size from 4.4 Mbp to 6.3 Mbp and GC contents were between 55-57%.

Sequence type

The study isolates were found to have 37 different STs, suggesting its diversity in India isolates. We found ST231 was predominant (n=43) among clinical strains. Followed by, ST147 (n=15), ST14 (n=15), ST2096 (n=11), ST395 (n=9), ST43 (n=8), ST 11 (n=7), ST23(n=6), ST16 (n=6), ST15 (n=3), ST437 (n=2), other clinical strains comprised individual STs, ST13, ST42, ST101, ST307, ST557, ST570, ST628, ST3249, ST711, ST660, ST3836, ST3835, ST3789, ST3607, ST3605, ST4847, ST2816, ST3249. The scatter plot represents the distribution of STs, where ST231, ST147, ST2096 emerged and displayed with increasing trends during 2013, 2015 and 2018 (Figure 1). Among these, ST14 was common across all years. The environmental strains comprised of individual STs namely, ST22, ST200, ST555, ST1107, ST1728, ST2701, ST3689 (Barrios et al., 2014). These STs, except ST1377 were also reported to be associated with clinical strains (Zhou et al., 2015).

Phylogenetic analysis

The WGS of study strains were used for the phylogenetic analysis based on MLST (Suppl Figure 1) and Single nucleotide polymorphism (Figure 2). The midpoint was divided into 2 major clades and further delineated into many clusters of *K. pneumoniae*. The environmental strain HPCN5, HPCN17, HPCN22, PL1-RCS238, AWD5, KBG6.2, EGD-HP19 were grouped in clade 1, whereas ME30 the endophyte isolate aligned in clade 2, suggesting the evolution as a separate ancestral group. The reference strain MGH78578 was closely aligned with environmental strain PVN1, STP sludge isolate. All the environmental strains were identified on a separate branch of the clusters consisting of clinical strains.

K. pneumoniae has a high prevalence of *β*-lactam and fluoroquinolone resistance

Using the ResFinder and CARD databases, 88 different predominant AMR genes involved in 8 antibiotic classes were selected for AMR profiling. The highest resistance found are fosfomycin 100% (n=153), β-lactam 96% (n=147), fluoroquinolone 92% (n=142) followed by aminoglycoside 84% (n=129), sulfonamide 79.08% (n=121), phenicol 70 % (n=107), macrolide 64.04% (n=98), rifamycin 48 % (n=74). AMR genes associated with fosfomycin (*FosA-like*), β-lactam (*blaCTX-M-15, blaTEM-1B*), fluoroquinolone (*oqxA-like, oqXB-like*) aminoglycoside (*aadA2, aac(6') Ib-cr-like*) were predominant across the India strains (Suppl. Table 2).

To understand the distribution of AMR genes in 153 genomes during 2010-2020, Box and whisker plot was used (Figure 3). The data revealed the presence of a genome having a least of one (n=1) and a maximum of 26 (n=4) AMR genes. The average median distributions of 15 AMR genes/genome were noticed during 2013-2017.

Most commonly, β-lactamase genes *blaCTX-M-15* (n=92), *blaTEM-1B* (n=87), carbapenamases *blaOXA-232* (n=66), metallo β-lactamase *blaNDM-5* (n=29) and *blaNDM-1*(n=10) were found among the study strains. *blaNDM-5* exhibited co-positivity with *blaCTX-M-15* and *blaTEM-1B*. An increasing trend in strains carrying *blaCTX-M-15*, *blaTEM-1B*, and *blaNDM-5* were observed from 2019.

Followed by, drug resistance mechanisms involved in study strains depicts antibiotic efflux (57.51%, n=88) and antibiotic inactivation by hydrolytic enzymes (41%, n=62) as predominant mechanisms. The major gene family observed for antibiotics efflux are resistance nodulation cell division (RND) (23 %, n=35), major facilitator superfamily (MFS) (18.95%, n=29) and ATP-binding cassettes (ABC) (15.68%, n=24). Similarly, in case of antibiotic inactivation, SHV β-lactamase gene family was prevalent (26.79% n=41) followed by, OXA- β-lactamase (6.9%, n=10) and CTX-M- β-lactamase (3.47%, n=5).

The predominant AMR gene in each 8 class of antibiotics was identified and screened its prevalence among 153 genomes. Such as, *blaCTX-M-15* and *blaTEM-1B* (β-lactam) *aadA2* (Aminoglycoside) *oqx-A-like and oqx-B-like* (Fluoroquinolone) *FosA-like*: (Fosfomycin), *erm(B)-like* (Macrolide), *sul1* (Sulfonamide), *ARR-2* (Rifamycin), *catA1like* (Phenicol). About 9.80 % of clinical isolates (n=15) were found to have all these 8 listed AMR genes, which mostly belong to ST231.

The environmental strains also carried the *blaSHV-1*, *FosA-like*, efflux pump mediated resistance (*oqx-A like, oqx-B-like*). Notably, the industrial effluent isolate carried *FosA-like, oqx-A-like, oqx-B-like, sul1, aac(6)Ib-cr-like, blaSHV112, ere (A)-like, aacA4-like, aadA1*.

IncF-type conjugative plasmid is dominant among *Klebsiella*

To understand the transmission of AMR determinants through plasmids, the type of plasmid and the resistance genes they carry were investigated. From the metadata, the number of plasmids estimated to be ranged from 1- 8 plasmids per strain, with most isolates harboring at least 2 plasmids. A clinical strain KpIMS38 isolated from pus harboured 16 plasmids. Followed by, the episome details of 153 strains were

obtained using Plasmid finder. A total of 19 plasmid replicon types were detected from 93.4 % (n=143) of isolates. The 19 plasmid replicon types are categorized as incompatibility plasmids (Inc) and mobilizable colicin plasmid (Col) groups. The most abundant epidemic plasmid is IncF-type conjugative plasmid (80.39 % n=123) (Figure 5). Plasmids belonging to IncF-type are reported to carry ESBL genes, genes encoding carbapenemases, aminoglycoside modifying enzymes and quinolone resistance genes (Rozwandowicz et al., 2018). The colKP3 plasmid was identified in 83 isolates which is related to *blaOXA-232* and *blaOXA-181*transfer. The second most prevalent were pKpQIL-IT plasmids (n=75) belonging to IncFIB (pQIL) family carries an aminoglycoside resistance gene on a transposon-like element IS26. Both colKP3 and pKpQIL-IT plasmid were accompanied in 33.9% (n=52) isolates. These two dominant plasmids were not detected in any of the environmental strains. IncHI1B (pNDM-MAR) plasmid with NDM resistance was found in 33 clinical strains and an environmental strain. The colistin resistance genes (*mcr1*) encoding plasmids (IncX4, IncY) were detected only in three strains among 153 study isolates. Certain resistant genes *blaCTX-M-15* (IncR, IncFII, IncFII, IncHI1B, Col440I), *blaNDM-5* (IncFII, IncX3) were detected in various plasmid replicon types. In the case of environmental strains, 5 isolates were harbored with one plasmid. The endophyte isolate has 2 plasmids possessing *blaCTX-M-15*, *blaNDM, qnrB1, blaSHV-56, FosA, oqxA* and *oqxB* resistant genes. The industrial effluent isolate has IncY, Phage-like plasmids encoding *mcr-1* gene, we have also recorded the multiple resistance being carried by this isolate. In summary, clinical and environmental strains have plasmids encoding genes that confer resistance to β-lactam drugs, aminoglycoside, quinolone and colistin. The type, family and functions of plasmids are discussed in Table 1.

Correlation of Resistance and Plasmid type

We narrowed it down to identify the strains that carry predominant AMR genes among the 8 selected antibiotic classes (n=15). These clinical isolates were found to have pKpQIL-IT and pKP3-A plasmid types. pKpQIL-IT carries an aminoglycoside resistance gene on a transposon-like element IS26, a clinically important insertion sequence. pKP3-A, a mobilizable but non-self-conjugative plasmid is associated with *bla-OXA-232* and *blaOXA-181* genes.

Associated virulence genes are higher in ST231 strains

The presence of different virulence genes is shown in (Figure 4), which were screened using VFDB. Both clinical and environmental isolates were found to have genes involved in type1 fimbriae (*fimD*, *fimK*, *fimH*, *fimC*). Similarly, most isolates were detected with *mrkD*, *mrkJ*, *mrkF*, *mrkC*, *mrkI*, *mrkA* which are important genes for type 3 fimbriae. To identify the hypervirulent phenotypes, *rmpA* a gene regulator for mucoid phenotype was analyzed. Nearly, 47% (n=77) of clinical and 33.3% (n=3) of environmental strains were identified as hypervirulent strains. Besides, the prevalence of *magA* gene (mucoviscocity associated gene) was comparatively less in clinical strains (n=56, 38.8%) than the *rmpA* genes. The *magA* was detected in environmental strains (n=5, 55.5%) as well. The presence of both *rmpA* and *magA* genes was not detected in root nodule, STP sludge and agriculture field soil isolate. The co-occurrence of *rmpA* and *magA* genes was observed in domestic sewage isolates (n=2) and an industrial wastewater isolate (n=1).

Likewise, the *rmpA* and *magA* were accompanied in clinical strains (n=38). These clinical strains were identified as blood isolates majorly with K51 serotype (n=18). Among the study strains, K51 (n=43) and K64 (n=33) serotypes were majorly found. Yet, the K51 serotype was not associated with severe infections and a recent report has identified K64 belonging to ST11 as an emerging superbug worldwide, however, none of the 153 Indian isolates were found to K64-ST11 (Zhao et al., 2020).

The most reported hypervirulent K1 and K2 serotypes were found in a smaller number of strains, n=7 and n=11 respectively (Brisse et al., 2013). The co-occurrence of *rmpA* and *magA* genes was observed in K1 (n=6) and K2 (n=2) serotypes belong to ST23 and ST14 respectively.

Followed by, most important siderophores were screened. The three most important siderophores systems in Enterobacteriaceae are yersinibactin (*ybtS*), aerobactin (*iutA*) and enterobactin (*entB*). Hence the prevalence of these siderophore systems was analysed in *K. pneumoniae* strains. The *entB* was detected in all the study strains (n= 153), *ybtS* gene was found in 98.6% in clinical (n=142) and 77.7 % in environmental strains (n=7) and *iutA* gene were observed in 97.2 % in clinical and 88.8% of environmental strains. The root nodule strain was negative for both *ybtS* and *iutA*. In addition, allantoin utilization (*allS*) was screened in the study strains as it has been associated with hypervirulence (Shon et al., 2013). The *allS* gene was observed in 65.97% of clinical (n=95) and 77.77 % of environmental strains (n=7). Further, the strains associated with hypervirulence factors (*magA, rmpA*, *allS*, *entB, ybtS, iutA*), were identified in 3 environmental strains (EGDHP90, HPCN17 and HPCN5) and 29 clinical strains. The number of associated virulence genes appeared to be high in strains ST231- K51 and ST23- KL1.

Convergence of AMR and Virulence

To investigate the relationship between AMR and virulence in *K. pneumoniae*, ESBL (*blaCTX-M-15*, *blaTEM-1B*) and carbapenamase (*blaOXA-232*) harboring isolates were mapped with major virulence genes (*rmpA, magA, ybtS, alls, iutA*). 98% of the ESBL and carbapenamase isolates harbored *ybtS and iutA,* 62% of isolates with *rmpA* and *magA.* The positive correlation between resistance and virulence in *K. pneumoniae* seeks prominent attention in clinical settings.

Discussion

This is the first comparative genomic study of *K. pneumoniae* isolates from India, which includes WGS of 144 clinical and 9 environmental strains collected during 2010-2020. Here, we examined antibiotic resistance, diversity of STs, serotypes, virulence genes, plasmids and their evolutionary relatedness.

Emerging drug resistant and virulent phenotypes of *K. pneumoniae* is a challenging concern worldwide. Prevalence of ESBL producing microorganism are increasing (Flokas et al., 2017; Kuralayanapalya et al., 2019). Nursing homes and intensive care units are the reservoirs of ESBL harboring microorganisms, mainly causing sepsis, urinary tract infections, peritonitis, intraabdominal abscesses, and upper respiratory infections (Sartelli et al., 2016; Lin et al., 2015; R. Podschun et al., 1998). The present analysis revealed the abundance of blood stream infections among Indian population in support with the previous

report (Wyres et al., 2019). As in line with the previous report, the AMR profile specified that fosfomycin (*FosA*) (Ito et al., 2017) is widely distributed among all study strains including environmental isolates (100%). However, the *in vitro* test demonstrates the susceptibility of fosfomycin, through *FosA* is widely found (Kopotsa et al., 2020). We observed second higher multidrug resistance to β-lactam antibiotics, most commonly carbapenem associated genes were detected. The prevalent ESBL types are SHV (sulfhydryl variable), CTX-M (cefotaximase) and TEM (Temoniera) (Parveen et al., 2012). Among these, CTX-M-type ESBLs are now found to be the most common type (Bradford, 2001). The *blaCTX-M-15* was widely distributed in clinical isolates since 2013 and increased overtime from 5.45% to 30.43% during 2017. As a result, *blaCTX-M-15* renders high resistance to large β-lactam agents (Moradigaravand et al., 2017). The OXA type ESBLs are found in 125 strains with the highest occurrence of *blaOXA232* (n=66). These *blaOXA232* producers belong to ST231 (n=31), ST14 (n=8), ST2096 (n=8). The coexistence of *blaNDM* and *blaOXA* was reported to have resistance to all β-lactam antibiotics (Naha et al., 2021). In the present analysis, we observed the associated *blaNDM* and *blaOXA* in 22.22% of clinical strain (Wyres et al., 2019). Further, among the study, we identified, 4 clinical strains were harbored with a high number of AMR genes (n=26). Two of these strains belong to ST14 and each of ST147 and ST437. These highly drug resistant strains harbored 4 important plasmid types of pK245 (*blaCTX-M-15*), pKP3-A (*blaOXA-232, blaOXA-181*), pKPN-IT (conjugative plasmid *aadA2, blaOXA-1, qnrB1, sul1*), pCROD2, (*MCR-1* carrying conjugative plasmid), pKpQIL-IT (aminoglycoside resistance gene on a transposon-like element IS26), pVM01 (hot spot bearing plasmid, *blaOXA-181*) suggesting the role of plasmid in multiple antibiotic resistance and transfer.

Capsular polysaccharide is the major virulence factor, which protects the bacteria from intracellular killing, phagocytosis, serum complement proteins and oxidative stress conditions (Marcoleta et al., 2018; Cortés et al., 2002). Looking across the Kaptive data set of 153 genomes, we found 29 serotypes among the *K. pneumonaie* isolates of which, K51 (28.1%) and K64 (21.56%) were majorly found. Most of the K51 isolates belong to ST231 (93.02 %) and K64, which are distributed diverse among major clonal types of ST14, ST147, ST395, ST2096 and ST231. Capsular types KL1, KL2, K5 K20 K57, are considered markers for hvKp strains (Shon et al., 2013; Yu et al., 2008). However, they are less prevalent among our study population (Suppl. table 3). In line with the previous report KL1 and KL2 isolates belong to ST23 (n=6) and ST14 (n=10) respectively. The environmental strains have identified with non-major serotypes such as KL16 (n=3), KL3 (n=2), KL116 (n=2), KL9 (n=1), KL166 (n=1). We then correlated the presence of *rmpA* and *magA* with capsular serotypes, where, K1 strains (n=7) were found to have both *rmpA* and *magA* (n=6) and five K2 strains were not detected with *rmpA* and *magA*. This observation, suggests that K1/K2 isolates may partially correspond to hypervirulence. Also, more than 50% of KL51 strains were found to have both *rmpA* and *magA*, recommending the hypervirulence characteristics of KL51.

Other chromosomally encoded pathogenicity factors that enables the bacteria to establish infection and progression of the disease by evolving immune evasion strategies were included. These include type1 fimbriae, type 3 fimbriae, type VI secretion system, type III secretion system translocator protein, type IV pilus secretion, Type IV pili, ferric aerobactin receptor, cytotoxic necrotizing factor 1, Allantion utilization (Parrott et al., 2021). Enterobactin (*EntB*), yersiniabactin (*YbtS*), and aerobactin (*iutA*) are the prevalent

siderophore among *K. pneumoniae* (Highsmith & Jarvis, 1985). They promote growth in the upper respiratory tract, lungs, and serum. *K. pneumoniae* producing both *ybtS* and *entB* can cause pneumonia. In our study, both *ybtS* and *entB* are prevalent among both clinical and environmental strains suggesting their role in the acquisition of nutrients from the environments. Also, allantoin utilization (*allS*) is considered an important virulence determinant of *K. pneumoniae* and characterized as hvKp that causes pyogenic liver abscesses (Chou et al., 2004s; Martin & Bachman, 2018). In our study population, *allS* is present in 95 clinical strains and 7 environmental strains. Similarly, the other virulence genes (type1 fimbriae, T3SS-translocator protein, fimbriae, PilQ, T4SS-pili) were present among all the study strains. The number of associated virulence genes (*rmpA, magA, entB, ybtS, iutA, allS*) appeared to be higher in ST231-KL51 isolates and ST23-KL1 isolates (Figure 6). Environmental strains (n=3) were found to have all the associated virulence genes. Discrimination of environmental and clinical strains is important in diagnosis & treatment. We categorize these strains as a true environment (root nodule, endophyte), and other may be originated from human or animals (domestic sewage, industrial wastewater, river surface water, STP sludge, rhizosphere soil, agriculture field). The genetic relationship of the isolates was analyzed based on SNP and MLST (Figure 6). Environmental isolates were out grouped. The MLST typing of environmental strains showed a diversity of sequence types, ST22, ST200, ST555, ST1107, ST1377, ST2701. These STs, except ST1377 were also reported to be associated in clinical strains (Zhou et al., 2015). Among these environmental strains, EDG-HP19, the industrial wastewater isolate was found to have a high number of resistant genes (AMR genes=9), when compared with other strains. However, we have noticed fewer AMR genes (< 10) in clinical strains as well (n=20). *oqx-A like, oqx-B-like and FosA-like* are commonly distributed among the 9 strains.

The environmental strains have been identified with non-major type serotypes such as KL16, KL3, KL116, KL9, KL166, KL50. The root nodule and domestic sewage isolates have KL5 serotype, which is also considered as a marker for hvKp. AWD5 and HPCN22 (n=2) were not detected with *rmpA* and *magA* genes. However, 49 clinical strains were also not detected with these two major virulent genes (Figure 6). Rhizosphere soil AWD5 *K. pneumoniae* is avirulent in the lung infection mice model (Rajkumari et al., 2021). As in previous reports, AMR and virulence characteristics are overlapped in both clinical and environment. Although the genomes of environmental strains collected in this study are not sufficiently conclusive to discriminate the *K. pneumoniae* from clinical settings and soil environments, some interesting observations were made. The phylogenetic analysis separates the environmental from clinical strains and we have noticed that the environmental strains are characterized by uncommon STs and serotypes. The plasmids, pKpQIL-IT and colKP3 dominant in clinical strains are not detected in environmental strains.

Conclusion

Taken together, our analysis with 153 genomes of *K. pneumoniae*, revealed the abundance of bloodstream infections among Indian population. Most commonly, β-lactamase genes *blaCTX-M-15*, *blaTEM-1B*and *blaOXA-232* were found among the study strains. *blaNDM-5* exhibited co-positivity with *blaCTX-M-15* and *blaTEM-1B*. An increasing trend in strains carrying *blaCTX-M-15, blaTEM-1B*, and

blaNDM-5 were observed from 2019. The OXA type ESBLs are found in 124 strains with the highest occurrence of *blaOXA-232*, majorly belong to ST231. Antibiotic efflux is the major drug resistance mechanism among the study strains. IncF conjugative plasmid is abundant followed by pKpQIL-IT and colKP3. These dominant plasmids are not found in environmental strains. The clinical isolates that have both *rmpA* and *magA* were identified as blood isolates majorly with K51 serotype. Diverse ST types were noticed among which, ST231, ST14, ST147 are prevalent. The number of associated virulence genes (*rmpA, magA, entB, ybtS, iutA, iutA, allS*) was found to be higher in ST231-KL51 and ST23-KL1 isolates, whereas the environmental strains are characterized by uncommon STs and serotypes. The convergence of resistance and virulence in *K. pneumoniae* is a major concern. Based on the present genomic investigations, we strongly suggest detection of *K. pneumoniae* strains with the genotype of ST231-KL51 and ST23-KL1 needs more attention for immediate treatment and preventing its dissemination.

Declarations

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Ethics Declaration

The study does not involve any ethical subjects.

Conflict of Interest

There is no conflict of interest.

Authors Contribution

All authors contributed equally to this work.

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Table

Table 1: The replicon type, family and function.

Plasmid	Family	Antibiotic resistant Genes	Functions of The Plasmid	References
pKpQIL-IT	IncFIB(pQil)	Aminoglycoside resistant gene	Plasmids capable of carrying transfer and MDR function	(Jousset et al., 2018)
pK245	IncR	*blaCTX-M-15*	Plasmid capable of carrying Carbapenenem resistance gene	(Dong et al., 2019)
pK2044	repB	*tetC, tetD*	conjugal transfer and maintenance	(Feng et al., 2017)
pVCM01	IncFII	*blaCTX-M-15, blaNDM-5, blaTEM-1* and *blaOXA-1*	conjugal transfer and maintenance	(Feng et al., 2017)
pKPX-1	IncFII(pKPX1)	*blaNDM-1, blaCTX-M-15, blaTEM-1, blaOXA-1*	conjugal transfer and maintenance	(Wysocka et al., 2021)
pKPN-IT	IncFIB(K)		conjugal transfer and maintenance	(Feng et al., 2017)
pNDM-MAR	IncHI1B(pNDM-MAR)	*blaCTX-M-15, blaNDM, qnrB1*	Limiting plasmid dissemination among potential host	(Newire et al., 2020)
pIncX-SHV	IncX3	*blaNDM–5*	Carrying various carbapenemase resistance genes	(Pál & Sonnevend, 2019)
pNDM-MAR	IncFI1B(pNDM-MAR)	*NDM-1*	Limiting plasmid dissemination among potential	(Newire et al., 2020)
pT5282-CTXM	IncX5	*blaKPC-5*	Dissemination	(Souza et al., 2019)
pNDM-KN	IncC	*blaCMY, blaNDM-1*	broad host range plasmids capable of carrying transfer and MDR function	(Ambrose et al., 2018)
pKPN3	IncFII(K)	*blaKPC-2*	Acquisition and dissemination of MDR	(Bi et al., 2018)
pCROD2	IncX4	*mcr-1*	MCR-1 carrying conjugative plasmid	(Bai et al., 2018)

P1 RepA (repA)	IncY	*mcr-1*	Phage- like plasmids	(Zhang et al., 2017)
pHN7A8	IncFII(pHN7A8)	*blaNDM-1*	Acquisition and dissemination of MDR	(Lin et al., 2017)
pMET-1 FC1	IncFII(pMET)	*blaOXA-48*	Acquisition and dissemination of MDR	(Perdigão et al., 2020)
pCAV1099-114	IncFIB(K) (pCAV1099-114)	*blaSHV-56, fosA, oqxA,* and *oqxB*	conjugal transfer and maintenance	(Maclean & Hanson, 2021)
pKPHS1	IncFIB(pKPHS1)	*KPC, OXA-48* and *NDM-1*	conjugal transfer and maintenance	(Wyres & Holt, 2018)
pBK30683	FIA(pBK30683)	*blaKPC-3*	Plasmids capable of carrying transfer and MDR function	(Liang Chen et al., 2014)
FDAARGOS_440	Col440I	*blaCTX–M–15, blaOXA–1, tet(B), tet(D), aac(6')-Ib-cr*	bla KPC bearing plasmids	(Yang et al., 2020)
pKP3-A	ColKP3	*bla- OXA-232, blaOXA-181*	Carries carbapenems resistance genes (oxa)	(Li et al., 2019; Shankar et al., 2020)
pBS512_2	Col(BS512)	*blaOXA-48, blaNDM-1* and *blaCTX-M-3*	Plasmid capable of carrying Carbapenenem resistance gene	(Tokajian et al., 2015)
pVCM01	ColpVC	*blaOXA-181*	Hot spot bearing plasmid(fitness)	(Oladeinde et al., 2018)

Figures

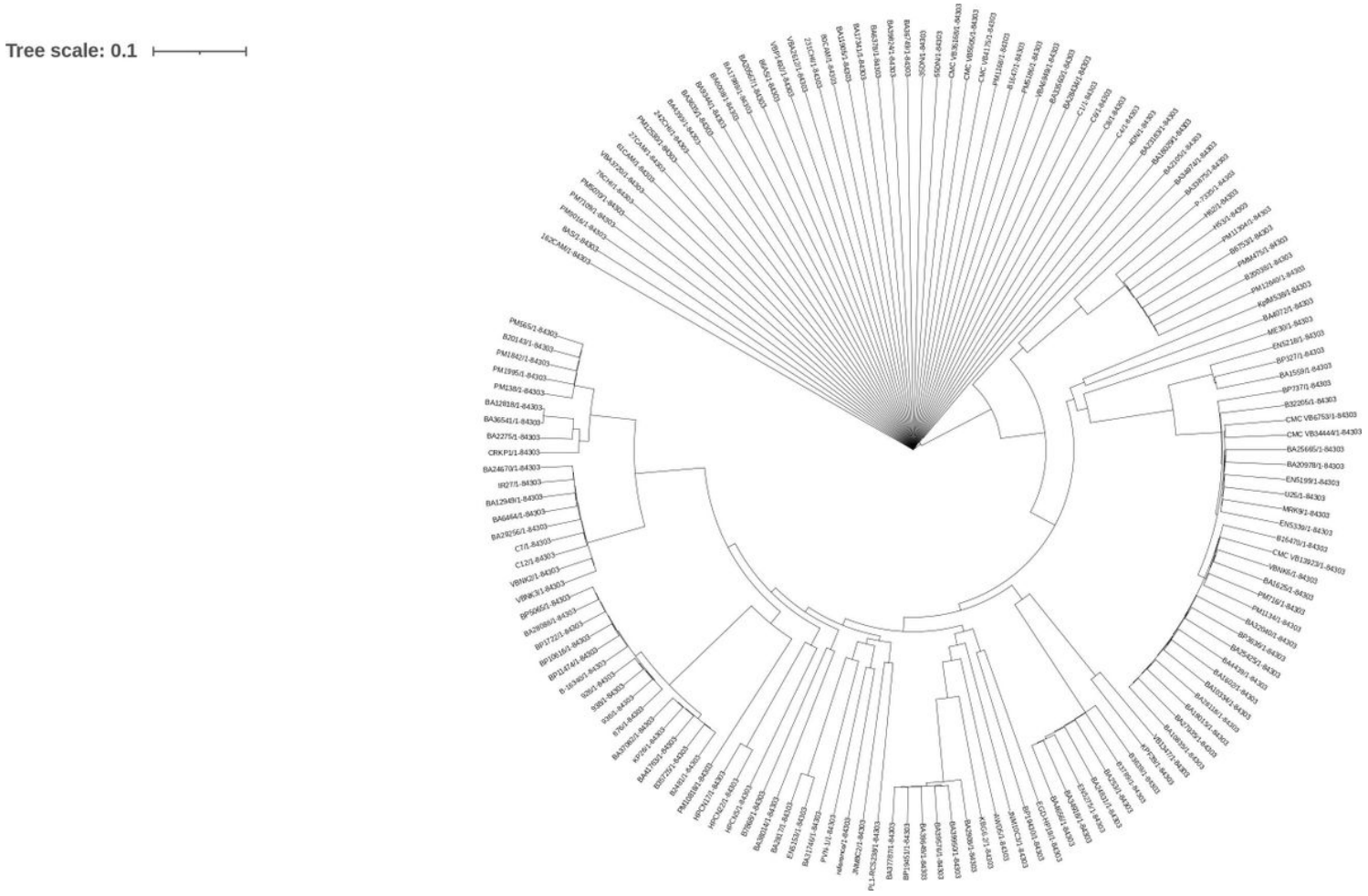

Figure 1

Year-wise ST distribution: The Scatter plot represents the distribution of STs across 2010-2020. ST231, ST147, ST2096 are displayed with increasing trends during 2013, 2015 and 2018. The points represent the STs representing the abundance from high to less. The line denotes the median points.

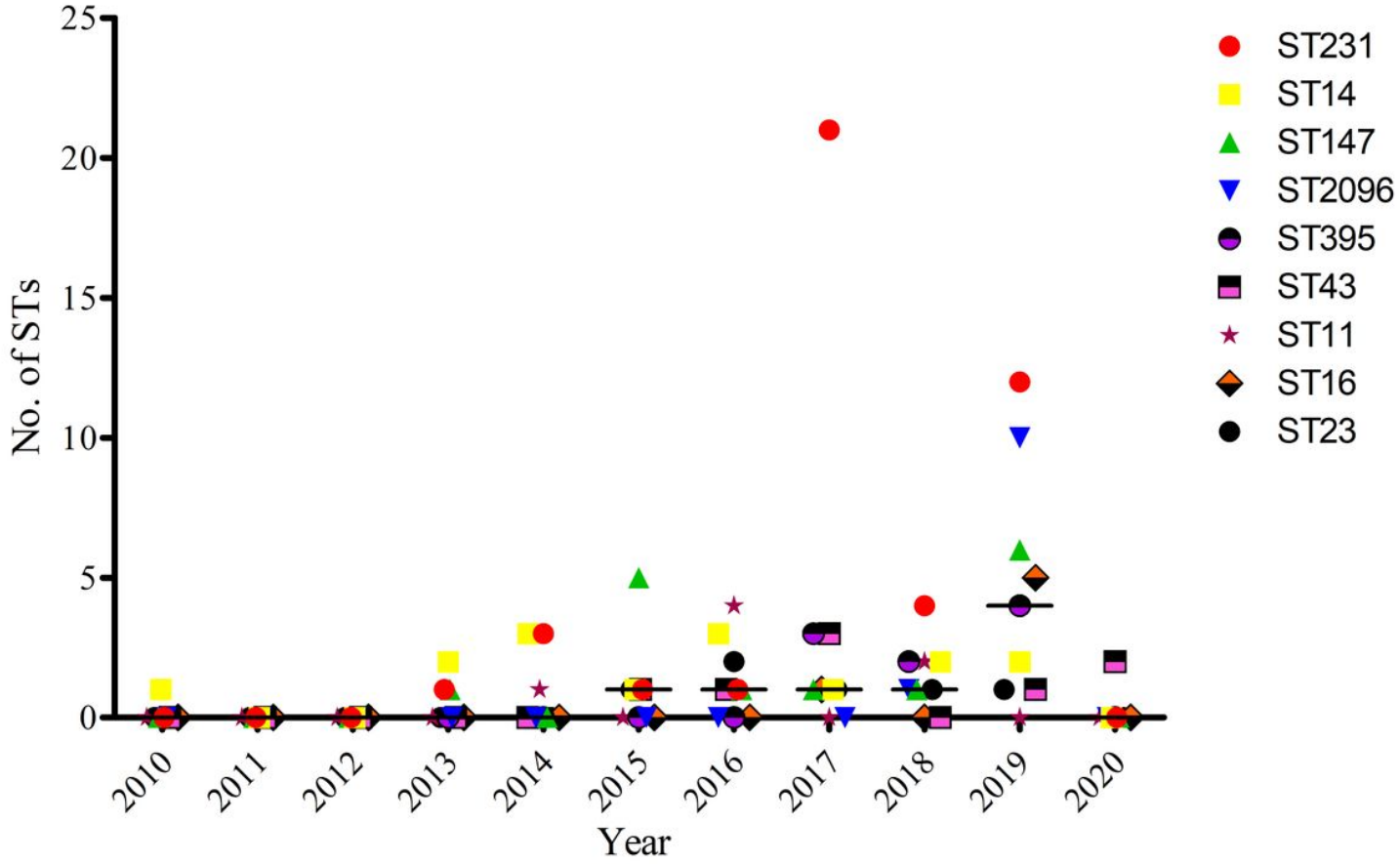

Figure 2

Phylogenetic tree based on SNP: SNP phylogeny was constructed by the neighbor joining method with MegaX. 1,000 bootstrap replicates were calculated to assess robustness. The midpoint of the phylogenetic tree was divided into 2 major clades and further delineated into many clusters of K. pneumoniae. The environmental strains were grouped in clade 1, whereas ME30 the endophyte isolate aligned in clade 2.

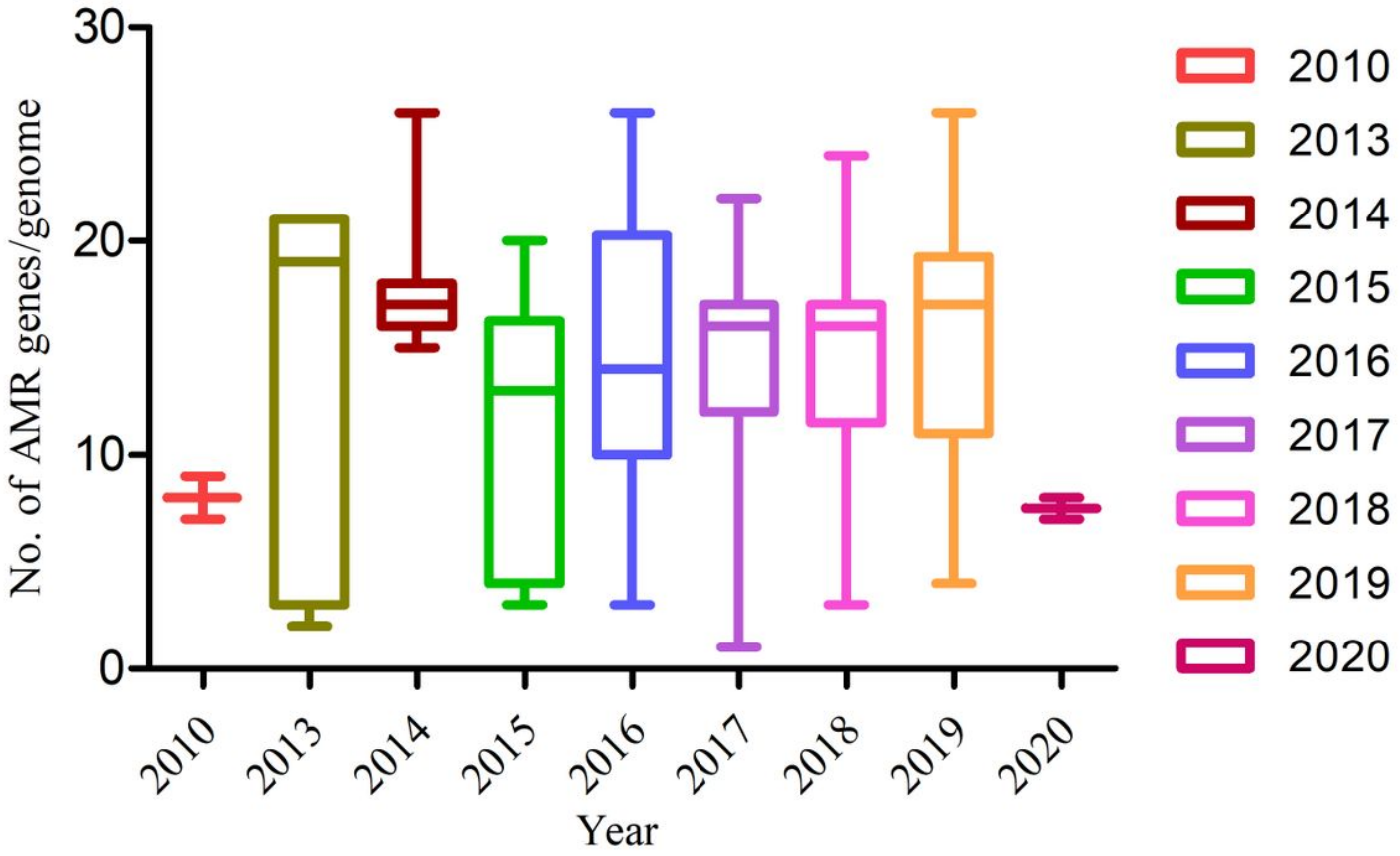

Figure 3

Distribution of AMR genes in K. pneumoniae isolates during 2010-2020: The Box plot represents the presence of one AMR and to the maximum of 26 AMR genes. The average median distribution of 15 AMR genes/genomes were noticed from 2013-2017. The bold horizontal lines represent the median. The whisker represents the upper and lower adjacent values.

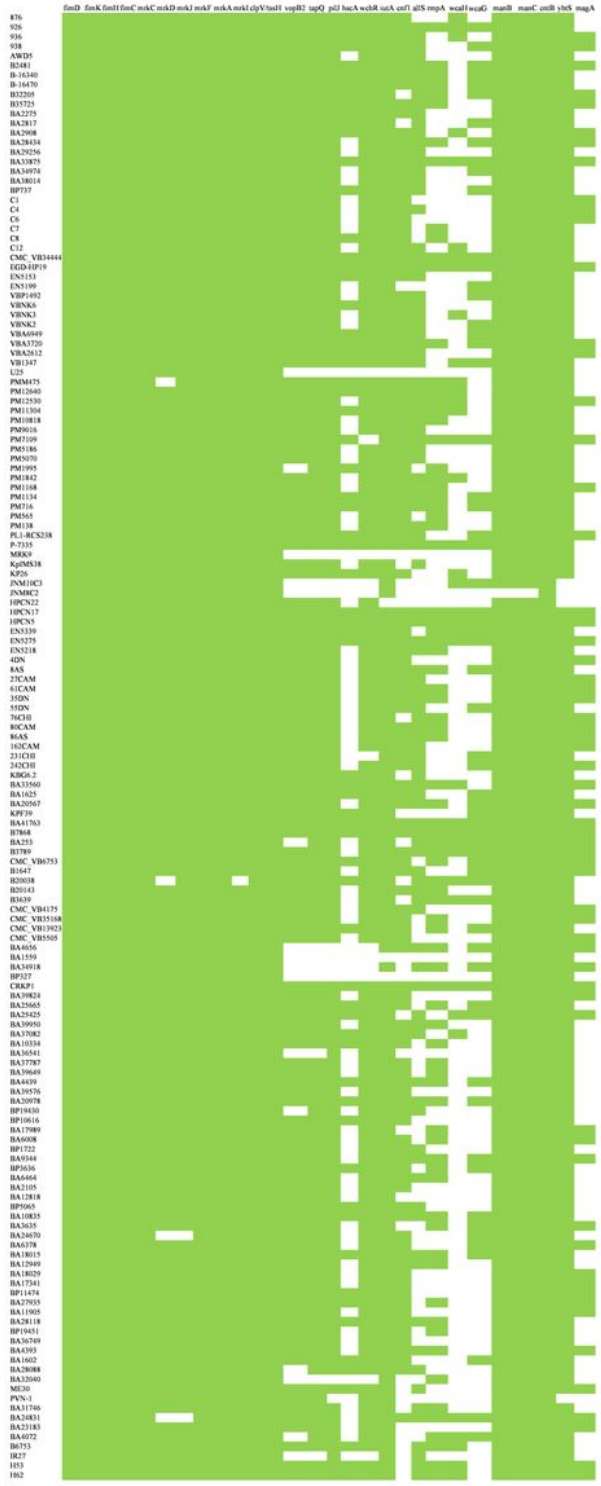

Figure 4

Virulome of K. pneumoniae: The virulence genes (n=27) in K. pneumoniae are screened using VFDB. The colour indicates the presence of virulence genes.

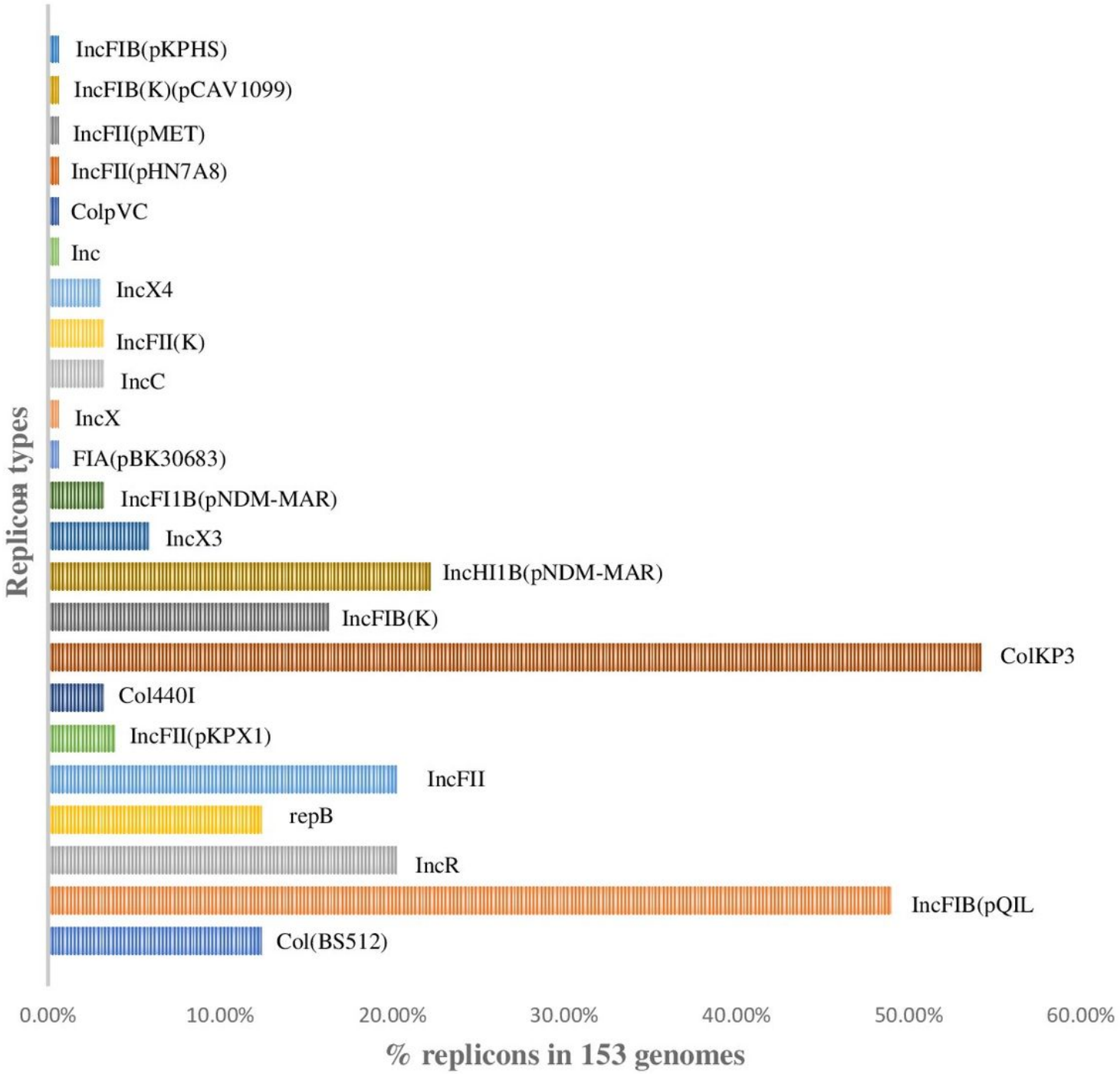

Figure 5

Prevalence of replicon types among the study strains: The bar graph corresponds to the percentage distribution of replicon types among 153 genomes.

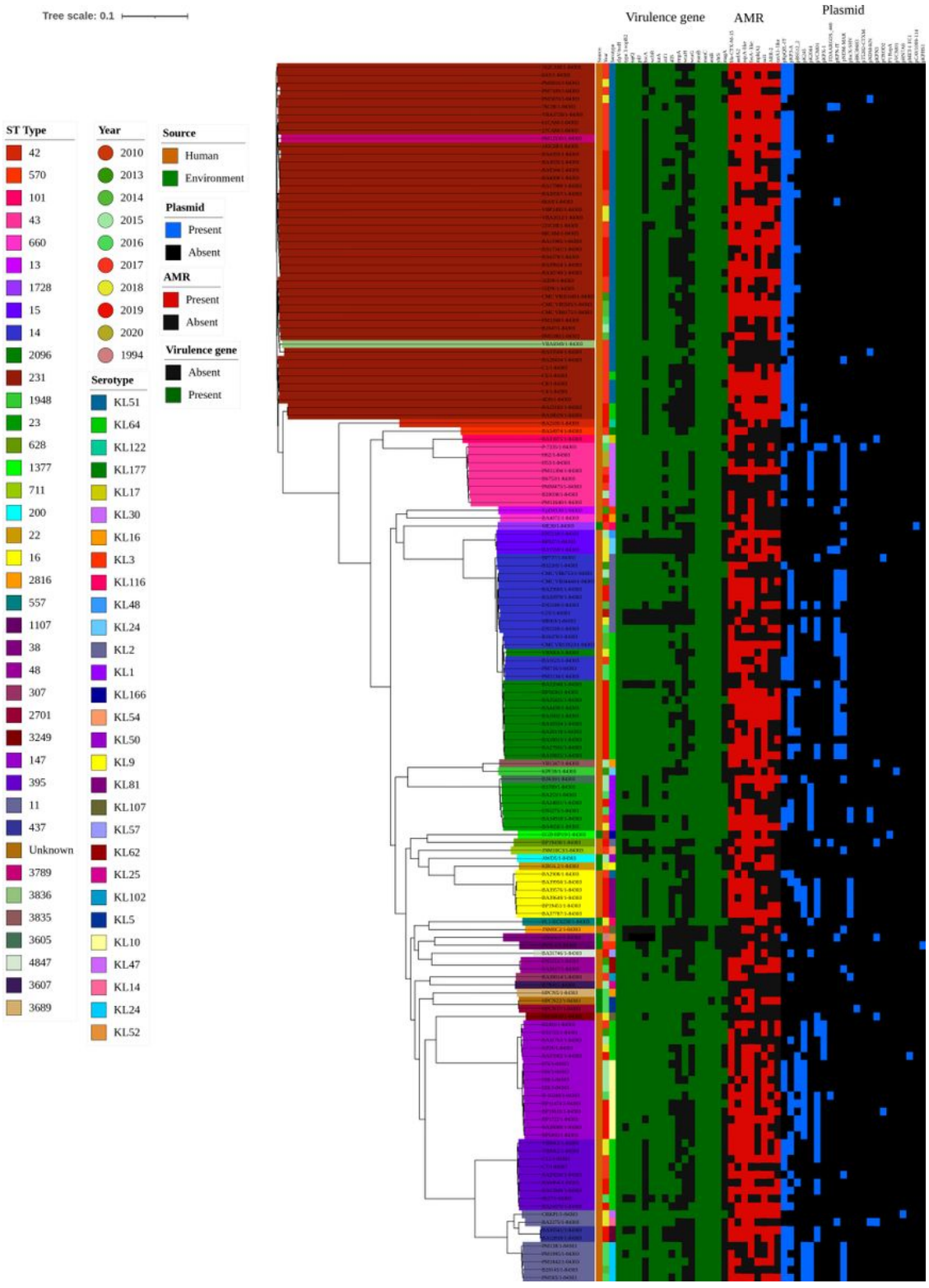

Figure 6

A complete picture of SNP based phylogeny: The tree consists of diverse STs and a few major STs that were more frequent in the clinical strains and uncommon STs are environmental strains. Colour of each branch denotes particular sequence typing. The isolation year, source, serotype is indicated by different colours. The presence of AMR genes, Virulence genes and replicon types are indicated.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplfig1.jpg
- Suppltable1.xlsx
- Suppltable2.xlsx
- suppltable3.xlsx
- suppltable4.xlsx