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Research Article

Biofilm Formation and Whole Genome Analysis of MDR *Klebsiella Pneumoniae* Strains Isolated from Hospital Acquired Infections in Tertiary Hospitals in Dakar, Senegal

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Abstract

Klebsiella pneumoniae is widely recognized as an opportunistic pathogen in both hospital and community settings. It is a key member of the ESKAPE group, which comprises priority microorganisms of major concern owing to their antibiotic resistance. The resistance of *K. pneumoniae*, particularly related to Extended-Spectrum β-Lactamases (ESBLs), poses a significant global public health challenge. The combination of its Multidrug Resistance (MDR) phenotype and various pathogenicity factors increases its potential to cause severe clinical infections. Biofilm formation was assessed via a semiquantitative microtiter technique. We employed various bioinformatics tools to analyze the Antimicrobial Resistance (AMR), virulence factors, plasmid replicons, and genomic diversity of the CRKP isolates. Overall, among the 24 *K. pneumoniae* isolates, most produced strong biofilms (n = 21), with some exhibiting moderate (n = 1) or weak (n = 2) biofilm production. An alarming level of resistance to multiple classes of antibiotics was correlated with the presence of various resistance genes, including those for β-lactams (bla_{OXA-48}, $bla_{OXA-181}$, $bla_{CTX-M15}$, bla_{TEM} and bla_{SHV}), aminoglycosides (*aph(6)-Id*, *aac(3)-IIe*, *aadA2*, *ant(3")-IIa*, *aph(3')-Ia* and *aac(6')-Ib-cr*), and quinolones (*qnrA*, *qnrB*, *qnrS*, *CRP*, and *emrR*). Various efflux pumps, such as *KpnGH, oqxAB*, *acrAB, acrD, and KpnEF*, are ubiquitously distributed across MDR *K. pneumoniae* strains. Several virulence-associated genes encoding type 1 fimbriae (*fimH*), type 3 fimbriae (*mrkA*), efflux pumps (*acrAB*, *oqxAB*), enterobactin (*entA*, *entB*, *fepC*), and yersiniabactin (*irp1*, *irp2*, *ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtT*, *ybtU*, *ybtX*) have been identified. Genetically, the isolates presented high diversity, with 18 Sequence Types (STs) and an average of 70.1% accessory genes. On the basis of SNP distance and pairwise ANI analysis, the majority of *K. pneumoniae* isolates were grouped into one clade. The high plasticity of *K. pneumoniae* in the acquisition of an MDR phenotype, combined with the phenotypic and genotypic factors described in this report, underscores the challenges in achieving effective clinical therapy with the available antibiotics. The findings also emphasize the critical need for the surveillance of multidrug-resistant pathogens in clinical settings in Senegal, as well as the need to evaluate their prevalence, propagation, and impact on patient health outcomes.

Keywords: *Klebsiella pneumoniae*; MDR; Biofilm; Resistance genes; Virulence genes; Plasmid replicons; ST; WGS; Bionformatics

Introduction

Klebsiella pneumoniae is an opportunistic pathogen responsible for a broad spectrum of Healthcare-Associated Infections (HAIs), predominantly affecting immunocompromised individuals and is responsible for diverse diseases syndromes such as pneumonia, bacteremia, urinary tract infections, wound or soft tissue infections, and liver abscesses [1]. *Klebsiella pneumoniae* ranks among the priority pathogens categorized within the ESKAPE-E group and is classified as a critical organism on the WHO priority pathogens list for the research and development of novel antibiotics [2]. Recent studies have highlighted this organism as one of the top five pathogens contributing to global mortality, regardless of its susceptibility to antibiotics [3]. In the USA, *K. pneumoniae* has been identified as a predominant cause of HAIs, accounting for an estimated 8.0% of all HAIs, whereas in the UK, it has been implicated in 4.7%–6.0% of all bacterial infections [4]. Sparse data from sub-Saharan Africa (sSA) suggest that *K. pneumoniae* may be responsible for higher proportions of HAIs in this region than in industrialized countries, particularly among children under 5 years of age [5-7]. In South Africa, *K. pneumoniae* caused 22.0% of HAI bacteremia cases among neonates, whereas in Kenya, it was estimated to be responsible for 20.0% of HAI bacteremia cases [8,9]. The emergence of Multidrug-Resistant (MDR) strains in *K. pneumoniae* is largely attributed to

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the acquisition of Antimicrobial Resistance (AMR) genes, which are commonly found among globally disseminated clones and often contribute to hospital outbreaks. Presently, *K. pneumoniae* resistance is predominantly associated with molecules such as third-generation cephalosporins and carbapenems. Various Extended-Spectrum β-Lactamases (ESBLs) responsible for resistance to third-generation cephalosporins have been identified in Senegal, including those from major bla_{CTX-M} groups such as $bla_{CTX-M15,}$ the most predominant [10-12]; $bla_{CTX-M109}$ [13]; bla_{SHV}-derived enzymes (bla_{SHV-2} and bla_{SHV-12}) [14]; and carbapenemases conferring resistance to carbapenems (e.g., bla_{KPC-2}, bla_{NDM} and bla_{OXA-48}) [15,16]. Additionally, resistance to fluoroquinolones, which is primarily mediated by Plasmid-Mediated Quinolone Resistance (PMQR) mechanisms [17,18], as well as modification enzymes conferring resistance to aminoglycosides [17], has been reported. Owing to the high adaptability of this pathogen, ESBLs, carbapenemases, PMQRs, and aminoglycoside-modifying enzymes may coexist in the same clinical strain, posing challenges for treatment options for affected patients [19].

Apart from AMR, the propensity of *K. pneumoniae* to cause severe infections is linked to virulence factors, biofilm formation and sequence types [20,21]. The determination of sequence types and clonal distributions is important, as certain clones, such as ST11 and ST258 or ST14, ST15, ST17, and ST37, are widely acknowledged for carrying MDR traits and have been linked to global outbreaks in human populations in recent years [22]. Virulence-associated genes, encompassing both fimbriae and nonfimbrial adhesins, iron-scavenging systems, and surface polysaccharides, play pivotal roles in the pathogenicity of *K. pneumoniae*. They are responsible for processes such as colonization, invasion, and pathogenicity of the strains [23,24]. One of the key virulence traits of *K. pneumoniae* is its ability to form biofilms, which are composed of bacteria enclosed within a self-generated extracellular matrix adhering to either living or nonliving surfaces [25]. This matrix is composed of proteins, exopolysaccharides, DNA, and lipopeptides [26]. Also, several virulence factors, including capsule polysaccharides, lipopolysaccharides, type 1 and type 3 fimbriae, outer membrane proteins, and mechanisms for iron acquisition and nitrogen utilization enable *K. pneumoniae* to survive, evade the immune system during infection, and contribute to biofilm formation [27,28].

In Senegal, there is a lack of whole-genome studies on AMR, which are essential for better understanding the mechanisms of resistance, virulence, and clonal distribution of isolates. This knowledge is crucial to strengthening AMR surveillance and evaluating available therapeutic options. This, the aim of this study was to give first insight of biofilm formation, resistome and virome among MDR-producing *K. pneumoniae* strains isolated from HAIs in Senegal.

Materials and Methods

Sample Collection

MDR *K. pneumoniae* strains were collected and processed from Hospital Aristide Le Dantec and the Children's Hospital Center Albert Royer of Fann Microbiology Laboratory from a previous study [29]. Bacterial strains resistant to at least three different antibiotic classes were classified as MDR, while those susceptible to only one or two antibiotic classes were categorized as XDR respectively, as previously described [30]. Infections were considered hospital-acquired if they developed at least 48 hours after hospital admission. Isolates were collected between January 2018 to February 2021. Antimicrobial susceptibility was evaluated by measuring strain growth zone diameters using the Kirby-Bauer method according to CA-SFM/EUCAST guidelines (version 2023) during a previous study [29]. The phenotypic resistance of the strains is given in *Table 1 supp.*

Hypermucoviscosity Characterization and Biofilm Formation Assay

The Hypermucoviscous (HM) phenotype was assessed via the "string test," following established protocols [31]. *Klebsiella pneumoniae* cultures were incubated on agar plates overnight at 37°C. A colony from the plate was subsequently stretched using a loop. If a viscous string formed, exceeding a length of 5 mm, the strain was classified as exhibiting the HM phenotype.

Biofilm production of MDR *K. pneumoniae* was assessed via a crystal violet staining assay. Briefly, the strains were grown overnight in Luria Bertani (LB) broth at 37°C under static conditions. Initially, 20 µL of the 0.5 McFarland bacterial standard and 180 µL of Luria–Bertani broth were inoculated into each well of a 96-well microplate, with six wells per strain, followed by incubation at 37°C for 24 hours. The Luria–Bertani broth was subsequently aspirated, and the wells were washed three times with Phosphate-Buffered Saline (PBS). The plates were stained with a 1% crystal violet dye solution (150 µL/well) for 15 minutes. After staining, the wells were washed three times with sterile water to remove unbound dye and then air-dried. The stained biofilms were solubilized with 150 µL of 100% ethanol for 10 minutes, and quantification was performed by measuring the optical density at 570 nm (OD_{575}). Each experiment was conducted in triplicate. The OD of the control wells with only media was used as the cutoff value (ODc). Using the ODc, the results of the biofilm formation assay were interpreted as follows: non biofilm producer (OD < ODc), weak producer (ODc < OD < 2ODc), moderate producer (2ODc < OD < 4ODc), and strong producer (4ODc < OD) [32]. Graphs and statistical analyses were performed via GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

DNA Extraction and Whole-Genome Sequencing

The genomic DNA of the *K. pneumoniae* isolates was extracted via the *PureLink™* Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Nucleic acid concentrations were measured via a Nanodrop spectrophotometer, and samples were adjusted to concentrations between 100 and 300 ng/µL. Whole-genome sequencing (WGS) was conducted via the NextSeq platform Illumina®. Dual-index sequencing libraries were prepared via the NEBNext® library preparation kit, Multiplex Oligos for Illumina® (NEB, Boston, MA, USA), and pooled. Sequencing was performed on an Illumina® Next 500 cartridge (2 × 150 bp).

Genomic Analysis

Genome assembly, annotation and sequence analysis: The quality of reads was conducted using FastQC [33]. The adaptor trimming was executed with fastp v0.23.2 [34] and the genomes were assembled from draft genomes with SPAdes v3.15.5 [35]. Finally, annotation was performed using Prokka [36]. AMR genes, virulence

genes and plasmid replicons were identified with the Comprehensive Antibiotic Resistance Database (CARD) [37], Virulence Factor Database (VFDB) [38], and PlasmidFinder [39], respectively, via Abricate v1.0.1 [40]. The approach proposed by Diancourt *et al.* [41] was employed for in silico Multilocus Sequence Typing (MLST), which involves evaluating allelic diversity across seven housekeeping genes (*gapa*, *infb*, *mdh*, *pgi*, *phoe*, *rpob*, and *tonb*). Whole-Genome Sequencing (WGS) data were utilized to identify the different sequence types (STs) and to determine the MLST profiles of the *K. pneumoniae* isolates via MLST v2.23.0 [42,43]. The K and O serotypes were determined via Kaptive 2.0 [44].

Phylogenomic analysis: To investigate the genetic diversity of the 24 MDR *K. pneumoniae* isolates in this study, pairwise Single Nucleotide Polymorphism (SNP) distances and pairwise Average Nucleotide Identity (ANI) values were analyzed via snp-dists v0.8.2 (*https://github.com/tseemann/snp-dists*) and FastANI v1.32 [45], respectively. Core genome alignment was performed via Roary [46] with a 95% minimum identity for BLASTX and a 99% core definition threshold. SNPs for each isolate were called from core genes using SNP sites v2.4.1 [47]. The phylogenetic tree was subsequently constructed employing gubbins [48], with the RAXML option for tree builder, and branch support was subsequently assessed using the neighbor-joining method with 500 bootstrap replicates. The resulting tree was visualized using Geneious [49]. The number of pangenomes was obtained, and a phylogenetic tree was visualized against a presence and absence matrix of the pangenomes via the roary_plots script (*https://github.com/sanger-pathogens/Roary/tree/master/contrib/roary_plots*).

Results

String Test and Biofilm Formation

The string test was negative, and none of the isolates were considered hypermucoviscous. Biofilm formation was detected in all strains, the majority of which were categorized as strong producers (n = 21), moderate producers (n = 1), or weak producers (n = 2) (Figure 1).

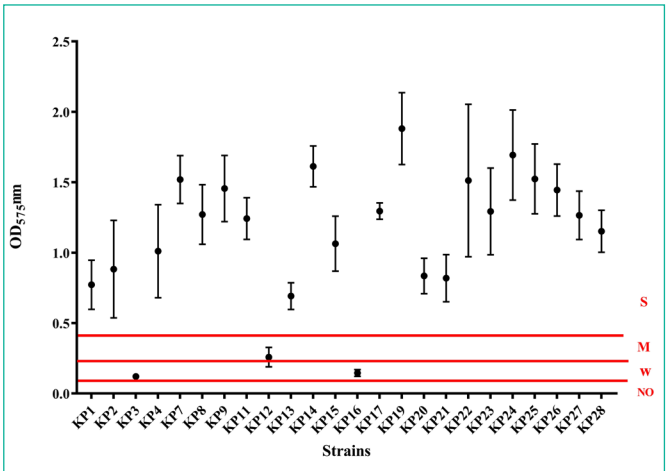

Figure 1: Biofilm formation of the 24 *K. pneumoniae* strains studied. The graphic shows the values (mean and standard deviation) of the optical density at 575 nm (OD_{575} nm) of crystal violet obtained for each strain. The dashed lines at 0.1, 0.21, and 0.42 represent the threshold value for each biofilm capability formation category: no biofilm Producer (NO), Weak (W), and Moderate (M) biofilm, respectively. Values above 0.42 were considered Strong (S) biofilm formation.

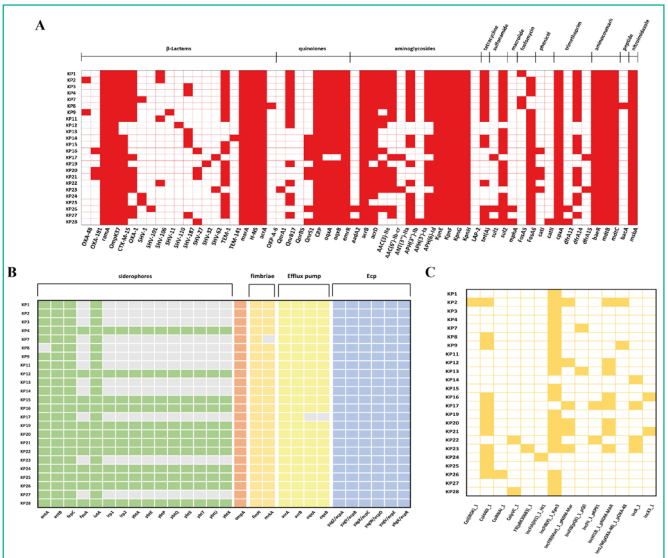

Figure 2: Distribution of A) Resistance genes. The white square indicates that the gene was not detected. B) Virulence genes of interest are shown in gray. ECP: *E. coli* common pilus. C) Plasmid replicons of 24 *K. pneumoniae* strains isolated from HAIs in Dakar.

Resistome, Virulome and Plasmid Analysis

For the resistome analysis, we identified carbapenem resistance genes, oxacillinases or class D β-lactamases, with bla_{OXA-48} found in 2 strains and its variant $bla_{OXA-181}$ in 3 strains. For the ESBL genes, $bla_{CTX-M15}$ was detected in 22 out of 24 strains. The bla_{OXA-1} ESBL gene was detected in 15 out of 24 strains. Multiple strains simultaneously harbored ESBL genes such as bla_{TEM} and bla_{SHV}. Additionally, the ESBL gene $bla_{PKP-A-6}$ was detected in one strain. Tetracycline resistance genes (*tetA*) and macrolide resistance genes (*mphA*) were detected in 14 and 2 strains, respectively. Various aminoglycoside resistance genes were also found: *aph(6)-Id* (20 strains), aac*(3)-IIe* (15 strains), *aadA2* (2 strains), *ant(3")-Iia* (2 strains) and *pph(3')-Ia* (2 strains). The *aac(6')-Ib-cr* gene, which confers resistance to both aminoglycoside and fluoroquinolones, was found in 15 out of 24 strains. Other important resistance determinants, such as quinolone resistance genes (*qnrA* (1/24), *qnrB* (14/24), *qnrS* (8/24), *crp* (24/24), and *emrR* (24/24)), efflux pump genes (*oqxAB* (23/24)), macrolide resistance genes (*mphA (2/24)*), trimethoprim resistance genes (*dfrA12* (2/24), *dfrA14* (17/24), *dfrA15* (2/24), and *cpxA* (24/24)), sulfonamide resistance genes (*sul1* (3/24) and *sul2* 23/24)), phenicol resistance genes (*catI* (4/24) and *catII* (1/24)), and fosfomycin resistance genes (*fosA5* (3/24) and *fosA6* (21/24)), were detected (Figure 2A, and Table S3).

Various types of efflux pumps are ubiquitously distributed across MDR *K. pneumoniae* strains, including major facilitator superfamily (MFS) antibiotic efflux pump genes (*KpnGH*), Resistance-Nodulation-cell Division (RND) antibiotic efflux pump genes (*oqxAB*, *acrAB, and acrD* (22 out of 24 strains)), and small multidrug resistance (SMR) antibiotic efflux pump genes (*KpnEF*). The porin OmpK37, which confers reduced susceptibility to β-lactams and carbapenems, was also detected in all the strains (Figure 2A).

Regarding virulence genes, those related to adherence, specifically the *E. coli* Common Pilus (ECP) genes (*ecpA*, *ecpB*, *ecpC*, *ecpD*, *ecpE*, and *ecpR*), type 1 (*fimH*), and outer membrane protein A (*ompA*), were present in all strains. Type 3 fimbriae (*mrkA*) were present in 23

		ST870	ST307	ST37	ST39	ST17	ST17	ST234	ST502	ST70	ST307	ST234	ST234	ST15	ST867	ST6	ST6	ST13	ST584	ST967	ST1243	ST39	ST20	ST1077	ST392
		KP1	KP11	KP12	KP13	KP14	KP15	KP16	KP17	KP19	KP2	KP20	KP21	KP22	KP23	KP24	KP25	KP26	KP27	KP28	KP3	KP4	KP7	KP8	KP9
ST870	KP1		732	831	778	722	755	771	752	692	746	781	779	769	828	688	714	733	803	769	726	798	768	4617	882
ST307	KP11	98.987		695	622	686	680	675	632	670	84	660	662	713	722	655	647	798	715	694	674	606	709	4692	687
ST37	KP12	99.05	99.044		704	685	738	698	710	745	711	690	696	744	728	711	710	759	708	697	691	690	730	4707	706
ST39	KP13	98.925	99.01	98.932		660	654	637	680	682	625	624	638	671	694	590	579	741	693	597	634	80	688	4652	712
ST17	KP14	99.01	99.081	99.028	99.097		253	660	666	687	690	657	673	706	722	644	633	702	586	722	661	659	293	4688	726
ST17	KP15	99.008	99.041	99.062	99.055	99.645		698	693	701	678	695	699	758	762	657	663	801	584	731	709	639	199	4768	745
ST234	KP16	98.991	99.039	98.953	98.952	99.071	99.069		658	684	661	57	47	727	737	657	663	781	718	754	675	642	708	4756	713
ST502	KP17	98.994	99.059	98.986	98.949	99.12	99.068	98.917		682	654	650	648	694	655	622	637	703	712	737	671	676	699	4695	722
ST70	KP19	99.035	99.167	99.038	98.98	99.016	99.072	98.946	98.963		667	675	692	716	723	690	684	686	758	725	680	684	750	4593	731
ST307	KP2	99.041	99.898	99.048	98.931	99.058	99.074	98.934	98.917	99.116		654	660	692	729	630	622	741	720	674	672	612	700	4679	691
ST234	KP20	99.032	99.042	98.939	98.954	99.076	99.055	99.929	98.911	98.966	98.991		41	713	726	644	639	742	722	730	670	628	700	4729	708
ST234	KP21	99.018	99.047	98.941	98.979	99.049	99.033	99.94	98.928	98.979	99	99.996		721	736	646	653	751	733	721	678	640	702	4729	720
ST15	KP22	99.14	98.997	98.978	98.91	99.093	99.059	99.015	98.916	98.957	99.061	99.028	98.998		732	618	610	759	760	839	705	676	771	4745	854
ST867	KP23	99.025	99.05	99.061	98.942	99.056	99.076	99	99.016	98.994	98.999	99.064	99.076	98.96		733	722	782	776	777	720	691	795	4731	732
ST6	KP24	99.09	99.086	99.013	98.977	99.009	99.043	99.14	98.98	98.991	99.09	99.133	99.122	99.115	99.01		69	735	670	694	662	587	686	4667	698
ST6	KP25	99.083	99.099	98.998	98.943	98.995	99.041	99.114	98.949	99.022	99.06	99.104	99.107	99.117	99.012	99.981		728	681	699	663	577	692	4641	709
ST13	KP26	99.09	99.068	99.01	98.938	99.052	99.081	99.055	98.98	98.989	99.022	99.017	99.013	99.112	98.931	99.125	99.096		745	759	758	726	897	4745	802
ST584	KP27	99.069	99.204	99.047	99.075	99.179	99.285	99.038	99.067	99.067	99.145	99.061	99.064	99.089	99.055	99.033	99.036	99.073		701	708	695	611	4714	710
ST967	KP28	99.018	99.085	99.011	99.164	99.096	99.095	99.077	99.081	99.036	98.945	99.075	99.059	99.057	99.042	99.038	99.034	99.06	99.056		730	590	751	4647	779
ST1243	KP3	99.097	99.075	99.02	98.948	98.949	99.069	98.984	98.973	99.001	99.061	98.998	98.99	99.084	98.995	99.084	99.102	99.127	99.098	99.051		637	714	4693	721
ST39	KP4	98.995	99.172	99.021	99.799	99.014	99.082	99.005	98.984	99.037	99.112	99.046	99.035	99.021	99.011	99.02	99.007	99.048	99.163	99.163	99.031		683	4649	721
ST20	KP7	98.973	99.041	98.979	98.943	99.514	99.693	98.954	99.023	99	98.941	98.989	98.977	98.977	98.983	98.969	98.953	99.028	99.237	98.921	98.982	98.984		4710	778
ST1077	KP8	93.892	93.888	93.838	93.743	93.835	93.814	93.825	93.833	93.822	93.835	93.781	93.791	93.872	93.832	93.876	93.905	93.803	93.843	93.764	93.877	93.881	93.788		4749
ST392	KP9	99.028	99.082	99.07	98.993	99.052	99.103	98.942	98.968	99.041	99.046	99.004	98.998	98.97	99	98.939	98.932	98.986	99.044	99.035	98.936	99.027	98.978	93.887	

ANI values
- > 95% (intra-species)
- 83-95%
- < 83% (inter-species)

Number of pairwise SNP distance
- 0-100
- 100-1000
- >1000

Figure 3: Matrix of pairwise Single Nucleotide Polymorphism (SNP) distances and pairwise Average Nucleotide Identity (ANI) values among MDR *Klebsiella pneumoniae* clinical isolates.

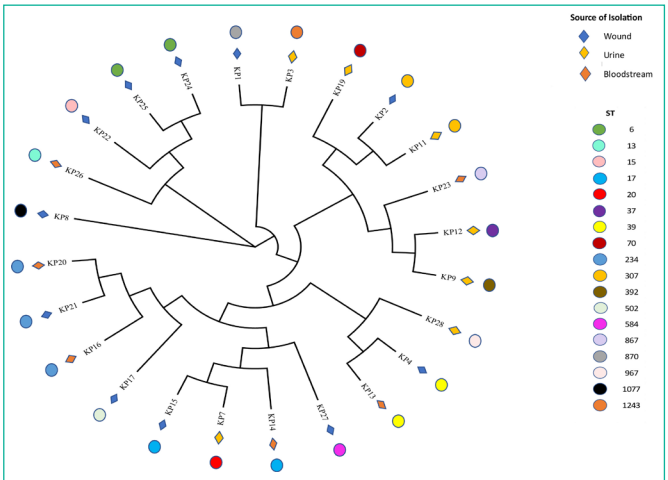

Figure 4: Phylogenetic tree of MDR *Klebsiella pneumoniae* clinical isolates based on single nucleotide polymorphisms.

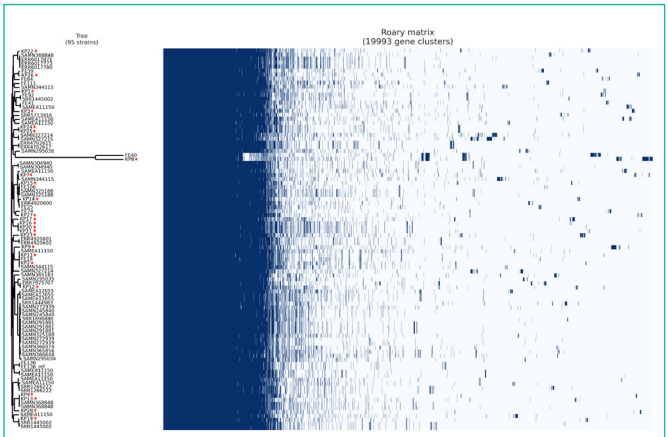

Figure 5: The single nucleotide polymorphism-based phylogenetic tree is shown against the pangenome matrix of MDR *Klebsiella pneumoniae* genomes compared with other published genomes.

out of 24 strains. The siderophore genes *entB* and *fepC* were also found in all strains, whereas *entA*, *irp1*, *irp2*, *ytbA*, *ybtE*, *ybtP*, *ybtQ*, *ytbT*, *ytbU*, and *ytbX* were present in some strains (Figure 2B and Table S4).

Fourteen plasmid replicons were detected in the studied isolates. They comprised five replicons from the IncF group, four from the Col group, and one each from the IncC, IncH, IncL, IncR, and IncX groups. Up to 6 plasmid replicons were found within the isolates. The IncFIB(K)_1_Kpn3 (n=20) and Col440I_1 (n=12) plasmid replicons were the most common in the *K. pneumoniae* isolates analyzed in this study, followed by IncHI1B_1_Pndm-MAR, IncFIB(Mar)_1_Pndm-Mar, and IncR_1, which were detected in 8, 6 and 4 isolates, respectively (Figure 2C and Table S5).

Genomic Diversity and Phylogenetic Tree

A matrix of pairwise SNP distances and pairwise ANI values is illustrated in Figure 3. The 23 K. pneumoniae isolates are grouped into one clade, with KP8 being relatively distant. The number of core SNPs within the clade varied from 42-882. Additionally, ANI values of 98.92-100% were found among all pairs of isolates, except for KP8, which presented an ANI of 93.83% compared with the other isolates. We identified 18 distinct STs (ST6, ST13, ST15, ST17, ST20, ST37, ST39, ST70, ST234, ST307, ST392, ST502, ST584, ST867, ST870, ST967, ST1077, and ST1243) among the 24 strains. The phylo-genetic tree analysis delineated separate clades on the basis of these STs (Figure 4 and Table S2). The comparison revealed 19,993 pan genes consisting of 5,988 (29.9%) core and 14,005 (70.1%) accessory genes. Among the accessory genes, 10,406 (74.3%) encoded hypothetical proteins (Figure 5 and Table S6).

Discussion

Gram-negative bacilli infections pose significant threats to hospitalized patients, with the potential to become life-threatening [50,51]. *Klebsiella pneumoniae*, an opportunistic pathogen, is associated with both community-acquired and nosocomial infections, causing pneumonia, abscesses, bacteremia, and urinary tract infections [52]. Its rapid acquisition of antimicrobial resistance

has escalated *K. pneumoniae* into a global concern, prompting efforts to curb the spread of multidrug-resistant strains [53]. A primary advantage of WGS lies in its ability to characterize the genomic content of clinically relevant bacteria, linking them to antimicrobial resistance and virulence-associated phenotypes. This enhances our understanding of their transmission within healthcare settings, facilitates accurate diagnostics, and enables prompt therapeutic interventions. This study aimed to characterize *K. pneumoniae* isolates from HAIs that occurred between 2018 and 2020 in Dakar, Senegal. Antibiotic exposure is a key driver of antimicrobial resistance, influenced by factors such as antibiotic use in healthcare, communities, agriculture, and the environment. Overuse, often due to easy access without prescription, contributes to resistance. In healthcare settings, prolonged and intensive antibiotic use is a major cause of the spread of resistant healthcare associated infections [54]. All Multidrug Resistance (MDR) *K. pneumoniae* strains in our study exhibited high resistance rates to commonly prescribed antibiotics, either individually or in combination. The observed resistance rates were as follows: β-lactams (100%), aminoglycosides (83.3%), fluoroquinolones (91.6%), cyclins (100%), fosfomycin (33.3%), and trimethoprim-sulfamethoxazole (91.6%). This finding is consistent with previous studies by Nirwati et al. [55] and Moini et al. [56], which reported that MDR *K. pneumoniae* isolates exhibited high levels of resistance to penicillins, cephalosporins, fluoroquinolones, aminoglycosides, and sulfonamides. A global meta-analysis of 47 studies estimated the prevalence of antibiotic resistance in healthcare-associated MDR *K. pneumoniae*. According to this meta-analysis, the resistance rates were as follows: β-lactams (91.5%), aminoglycosides (85.1%), quinolones (87.2%), cyclins (34%), sulfonamides (51%), polymyxins (14.9%), and other classes of antibiotics (38.3%) [57].

All of the strains were ESLB-producers and $bla_{CTX\text{-}M15}$ was found as the main ESBL gene (22 out of 24, 91.6%) in our *K. pneumoniae* isolates confirms that CTX-M-15 is currently the most widely distributed CTX-M enzyme in Senegal [58,59] and worldwide [60]. A study conducted in medical biology laboratory at Institut Pasteur Dakar showed that a majority of ESBL-producing *E. coli* strains were sensitive to cefoxitin and piperacillin-tazobactam suggesting that these antibiotics can be used as alternatives to carbapenems in the treatment of ESBL-secreting *Enterobacteriaceae* infection [58]. Additionally, the presence of various ESBL genes, such as bla_{TEM} (18 out of 24, 75%) and SHV (23 out of 24, 95.8%), underscores the potential role of *K. pneumoniae* as a reservoir for beta-lactam and non-beta-lactam resistance determinants, posing major concerns in countries with inadequate antibiotic resistance surveillance, prevention, and containment measures [61].

Among them, 8 *K. pneumoniae* strains were resistant to carbapenems, with the $bla_{OXA\text{-}48}$ gene detected in 2 strains and $bla_{OXA\text{-}181}$ in 3 strains. For the remaining carbapenem-resistant strains, the presence of various efflux pumps (*ramA-acrAB*) and a porin system (*OmpK37*) was observed, which can decrease susceptibility to beta-lactam antibiotics, including carbapenems [62,63]. The widespread of multidrug resistance mechanisms of *K. pneumoniae*, especially the global spread of carbapenemases, combined with the rapid increase in carbapenem consumption in LMICs are driving increased carbapenem resistance especially in the ICU where they are the leading causes of invasive HAIs [64].

In nearly all the isolates investigated, genes associated with resistance to aminoglycoside, trimethoprim, sulfonamide, tetracycline, and chloramphenicol were found, which correlates with antibiotic susceptibility results.

In addition to AMR genes, our *K. pneumoniae* isolates harbored several virulence-associated genes, including those encoding type 1 fimbriae (*fimH*), type 3 fimbriae (*mrkA*), efflux pumps (*acrAB*, *oqxAB*), enterobactins (*entA*, *entB*, *fepC*), and yersiniabactin (*irp1*, *irp2*, *ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtT*, *ybtU*, *ybtX*). Previous studies have demonstrated a significant association between *mrkA* and biofilm formation *in K. pneumoniae* [65,66], while *fimH* has been strongly linked to the MDR phenotype [65,67].

Biofilms are microbial communities that are encased in a matrix that maintain bacterial structural integrity, can attach to both biotic and abiotic surfaces and protect bacterial cells against antibiotics and the host's immune system [68]. The majority of the strains exhibited a strong biofilm formation phenotype (22/24, 91.6%) and several studies have reported strong biofilm production in the majority of clinical *K. pneumoniae* strains [55,69,70]. In our study, biofilm production correlates with the presence of type 1 (*fimH*) and type 3 fimbriae (*mrkA*), both of which are crucial for adhesion to host cells [71]. These fimbriae also play a significant role in biofilm formation across many species [72]. *fimH* has been implicated in adhesion to epithelial cells, colonization, biofilm formation, and immune evasion [73], whereas *mrkA* is crucial for binding to host cells and extracellular matrix proteins, promoting biofilm formation on both biotic and abiotic surfaces [74]. The Minimum Inhibitory Concentrations (MICs) of conventional antibiotics for biofilm bacteria are 100–1000 times higher than those for planktonic bacteria [75] and biofilm-producing ability have been shown to correlate with extensively drug resistant (XDR) *K. pneumoniae* antibiotic resistance profile [76]. This inherent tolerance to antimicrobial agents, can then lead to severe, persistent infections that are particularly difficult to treat particularly in hospital settings [77].

The overexpression of the multidrug efflux pump *acrAB* in gram-negative bacteria not only confers resistance against antibiotics such as fluoroquinolones, β-lactams, and tigecycline but also provides virulence factors, such as resistance to antimicrobial peptides produced by the innate immune system in the lungs [78,79]. The *oqxAB* multidrug efflux pump mediates resistance in various bacteria, especially *K. pneumoniae* and *E. coli*, and can be found on both chromosomes and plasmids [80,81]. Previous studies have demonstrated that *oqxAB* confers resistance to fluoroquinolone, olaquindox, tigecycline, nalidixic acid, and chloramphenicol [80-83]. Siderophores such as enterobactin and yersiniabactin facilitate iron uptake and protect microorganisms against oxidative stress from host innate immune cells, thereby promoting infection [84,85].

The persistence of carbapenem resistance genes is driven primarily by the clonal dissemination of isolates and the spread of these genes via conjugative or mobile plasmids [86]. In this study, we identified five types of Inc. plasmids known to facilitate the spread of the $bla_{OXA\text{-}48}$ and bla_{NDM} genes in *K. pneumoniae* isolates. All the detected IncL plasmids harbored $bla_{OXA\text{-}48}$ resistance genes, while two plasmid replicons carrying bla_{NDM} genes were identified. Although these particular plasmids did not carry the bla_{NDM} gene, their presence

suggests the potential for these strains to acquire it. IncFIB and Col were the most frequently detected plasmids. The IncFIB gene is a conjugative plasmid previously associated with the dissemination of the bla_{TEM} gene in *E. coli* isolates from Africa and is responsible for the spread of the bla_{NDM-1}, bla_{SHV-12}, $bla_{CTX-M15}$, and bla_{OXA-1} genes in *K. pneumoniae* in Europe [87,88]. This study is the first to report these plasmid replicons in clinical *K. pneumoniae* isolates from Senegal. The detection of these multiple plasmid profiles in our strains raises concerns about the rapid spread of antibiotic resistance in hospital settings, with the likelihood of these plasmid types becoming more prevalent in the future.

Among the 24 strains, 18 different sequence types were identified, indicating significant diversity among the clinical HAIs in Dakar. The variation in ST distribution across different study locations highlights the genetic diversity of this pathogen, with majority of the STs being widely dispersed [89,90]. Additionally, our analysis of the genetic relatedness of the strains revealed moderate pairwise SNP distances in pairs of different STs, whereas > 1000 SNPs were identified in ST1077 compared with the other STs. The commonly applied

ANI threshold of 95-96% supports species assignments [91]. Our study revealed that all MDR strains shared greater than 97% ANI, suggesting that they were closely related strains of *K. pneumoniae* with similar gene presence/absence patterns [92]. However, KP8 (ST1077) shared less than 94% ANI with other *K. pneumoniae* isolates, confirming that it is a relatively distinct ST from its closest phylogenetic neighbors. *K. pneumoniae* ST1077 is closely related to ST1224 that was isolated from dairy products and chicken meat, respectively, in libya and Western Algeria, suggesting that this Klebsiella isolate is not host-specific and could be easily transmitted to humans from food animals and their products [93,94]. Pangenome analysis revealed substantial diversity, with a high percentage of accessory genes (70.1%) and a significant proportion of hypothetical proteins (74.3%) among these accessory genes

K. pneumoniae is thought to possess an open pangenome due to its ubiquity across diverse environments, including mammalian guts, soils, and surfaces, where it can potentially exchange genetic material with other bacterial species [95]. These findings suggest the broad diversity, widespread dissemination, and rapid adaptive evolution potential of MDR clinical *K. pneumoniae* strains in Senegal.

Conclusions

In this study, we present genomic insights into MDR *K. pneumoniae* clinical isolates from tertiary university hospitals in Dakar. These isolates exhibit strong biofilm formation ability, which could contribute to their persistence in hospital environments. They also belong to various sequence types and carry multiple antimicrobial resistance genes, virulence genes, and plasmid replicons. Through analysis of SNPs, ANI, and phylogenetic data, the isolates were found to be primarily clustered into a single major clade. Our findings elucidate the genomic characteristics and pathogenic traits of these clinical isolates. Pangenome analysis revealed significant genomic plasticity, suggesting the potential for the evolution and dissemination of these pathogens. We recommend search for alternative antibiotic treatment options, especially for carbapenem-resistant *Enterobacteriaceae* among clinicals isolates in Senegal, as well as novel therapeutic approaches such as bacteriophages for difficult-to-treat and biofilm-associated infections. We also advocate reinforcing AMR surveillance, implementing antimicrobial stewardship policies, and enhancing infection control measures in hospitals to reduce the selective pressure driving the emergence and spread of MDR strains.

Author Statements

Author Contributions

Conceptualization: IN, GCDM and AS; Methodology, IN, OS, AC, BSB; Software, IN; Validation: GDCM and AS, Formal Analysis: IN; Bioinformatic and phylogenetic analysis, IN; Validation, GCDM and AS; Investigation: IN, MMB, AD and AD; Resources: MMB, AD, AD, BD, FT, OS, AC, BSB, CF, YD; Data Curation: IN; Writing – Original Draft Preparation: IN.; Writing – Review & Editing: BD, FT, OS, AC, BSB, CF, YD, GCDM and AS; Visualization: IN; Supervision: GCDM and AS; Project Administration: GCDM and AS.

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Conflicts of Interest

The authors declare no conflicts of interest.

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