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High prevalence of *bla*_{CTX-M-15} and nosocomial transmission of hypervirulent epidemic clones of *Klebsiella pneumoniae* at a tertiary hospital in Ethiopia

Tsegaye Sewunet^{1-3*}, Daniel Asrat¹, Yimtubezinash Woldeamanuel ¹, Sofia Ny⁴, Fredrik Westerlund ⁵, Abraham Aseffa⁶ and Christian G. Giske^{3,7}

¹Department of Microbiology, Immunology and Parasitology, Addis Ababa University, Addis Ababa, Ethiopia; ²School of Laboratory Sciences, Jimma University, Jimma, Ethiopia; ³Department of Laboratory Medicine, Division of Clinical Microbiology, Karolinska Institutet, Stockholm, Sweden; ⁴Public Health Agency of Sweden, Stockholm, Sweden; ⁵Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden; ⁶Armauer Hansen Research Institute, Addis Ababa, Ethiopia; ⁷Karolinska University Hospital, Clinical Microbiology, Stockholm, Sweden

*Corresponding author. E-mail: tsegishs2010@gmail.com; tsegaye.sewunet@ki.se

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Background: Genomic epidemiology of antibiotic resistance is not sufficiently studied in low-income countries.

Objectives: To determine prevalence of ESBL production, and resistome and virulome profiles, of *Klebsiella pneumoniae* isolated at Jimma Medical Center, Ethiopia.

Methods: Strains isolated from patients with suspected infections between June and November 2016 were characterized by MALDI-TOF for species identification and disc diffusion for antimicrobial susceptibility testing. All *K. pneumoniae* isolates were characterized by double disc diffusion for ESBL production and all ESBL-producing strains (ESBL-KP) were subjected to WGS on the Illumina (HiSeq 2500) platform. DNA was extracted by automated systems (MagNA Pure 96). Genome assembly was performed using SPAdes (v. 3.9) and draft genomes were used for analysing molecular features of the strains. Maximum likelihood trees were generated using FastTree/2.1.8 based on SNPs in shared genomic regions to identify transmission clusters.

Results: Of the 146 *K. pneumoniae* strains isolated, 76% were ESBL-KP; 93% of the ESBL-KP strains showed resistance to multiple antimicrobial classes. *bla*_{CTX-M-15} (84.4%) was the most prevalent ESBL gene. Resistance genes for aminoglycosides and/or fluoroquinolones [*aac*(6')-Ib-cr (65.1%)], phenicols [*catB3* (28.4%)], sulphonamides [*sul1* (61.2%) and *sul2* (60.5%)], trimethoprim [*dfrA27* (32.1%)], macrolides [*mph*(A) (12.8%)] and rifampicin [*arr2/arr3* (39.4%)] were prevalent. Plasmids of the IncF and IncR families were prevalent among ST218, ST147, ST15 and ST39. KL64 and KL57 capsular types and O1 and O2 LPSs were prevalent. A high-risk clone, ST218-KL57 encoding *rmpA1/rmpA2* and *iutA*, was detected. Phylogenetic analysis showed a cluster of clonally related strains from different units of the hospital.

Conclusions: Prevalence of ESBL-KP was high and *bla*_{CTX-M-15} was the predominant ESBL gene. ESBL genes had spread through both clonal and polyclonal expansion of high-risk and hypervirulent clones. Nosocomial transmission of MDR strains between different units of the hospital was observed.

Introduction

Enterobacteriaceae are widely distributed in nature and are frequently isolated from infected patients, animals and the environment. In concordance with their abundance in nature, *Escherichia coli* and *Klebsiella pneumoniae* are frequently isolated from clinical samples. *K. pneumoniae* is most prevalent in hospitals among

admitted patients and is often identified with more resistance to antimicrobials than *E. coli*. *K. pneumoniae* causes different types of infection among immunocompromised individuals, children and elderly people.^{1,2}

Though *K. pneumoniae* is largely recognized as a cause of hospital-acquired infections, hypervirulent strains can also cause serious infections among apparently healthy individuals.³ These

hypervirulent strains are often resistant to antimicrobials commonly prescribed for infections caused by Gram-negative bacteria. These antimicrobials include extended-spectrum cephalosporins, carbapenems, aminoglycosides, fluoroquinolones and polymyxins. Currently, ESBL-producing *K. pneumoniae* (ESBL-KP) has become a challenge in healthcare in every country in the world (<https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/>), and high-risk and hypervirulent clones of *K. pneumoniae* are increasingly prevalent.^{4,5}

The presence of a large accessory genome and the genomic plasticity of *K. pneumoniae* to acquire both resistance and virulence genes have been fairly well understood. The increased prevalence of antimicrobial resistance (AMR) and virulence in *K. pneumoniae* strains is to a large extent due to acquisition of mobile genetic elements.⁶ Furthermore, an increased fitness of successful clones and continuous exchange of genetic determinants for AMR may lead to pan-resistant clones of *K. pneumoniae*.³ Global efforts of tackling AMR consider antimicrobial surveillance strategies that cover screening for high-risk clones, however, genomic epidemiology and surveillance studies from low-income countries are limited. On top of a compromised economy, an increased burden of AMR in low-income countries is a real threat to healthcare systems.

While few phenotypic studies show high prevalence of AMR, genomic epidemiology of ESBL-producing Enterobacterales was not previously studied in Ethiopia. We here characterized the prevalence of ESBLs, molecular features, STs and virulence genes in *K. pneumoniae* strains isolated from suspected infections at Jimma Medical Center, Ethiopia. Moreover, we analysed SNPs between strains isolated at different units of the hospital to determine potential nosocomial transmission of strains within the hospital.

Methods

A cross-sectional study was conducted among patients seeking medical care at Jimma Medical Center in Jimma, Ethiopia; a 600-bed hospital with 13 units serving a total population of 15 million people. Non-repeat single specimens were collected from each patient with suspected infection (pneumonia, diarrhoea, wound infection and urinary tract infection) from June to October 2016. Data regarding sociodemographic variables and putative risk factors (presence of underlying chronic illness, admission, and current usage of antibiotics) were collected using a structured questionnaire. Strains were isolated by conventional biochemical methods at the study site.

All *K. pneumoniae* isolates ($n = 146$) were tested for ESBL production phenotypically by the double disc diffusion test (DDST) using cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and amoxicillin/clavulanic acid (30 µg, 20/10). Species identification was further validated by MALDI-TOF, and antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion technique according to EUCAST guidelines (https://www.eucast.org/ast_of_bacteria/). All ESBL-KP strains ($n = 111$) were subjected to WGS. After overnight aerobic incubation on cystine lactose electrolyte deficient agar at 37°C, 2–5 pure colonies were taken with inoculating loop and were then suspended in BSA (0.5 mg/mL) in PBS (pH 7.4). Then, the cell suspensions were aliquotted in a 96-well plate and genomic DNA was extracted using an automated DNA extraction system (MagNA Pure 96, Roche Life Sciences, Sweden). DNA concentrations were measured with Qubit™3.0 (Thermo scientific, MA, USA). Sequencing libraries were generated using Nextera XT (Illumina kits) and short-read sequencing was

run on Illumina (HiSeq 2500) systems with a 150 bp insert size paired end sequencing protocol at Science for Life Laboratory, Stockholm, Sweden.

SPAdes (version 3.9) was used for genome assembly. The assembled draft genomes were used for analysis of resistome and virulome, as well as plasmid replicon and sequence typing using CGE tools (<http://www.genomicepidemiology.org/>). For virulence genes and other genomic analysis BIGSdb (<https://bigsdatabases.pasteur.fr/index.html>), a *K. pneumoniae* genome analysis pipeline hosted at Institut Pasteur, was used. Kaptive/Holt Lab (<https://kaptive-web.erc.monash.edu>) was used to predict capsular types and O-LPSs. For all databases, the putative genes were considered positive when the coverage and the percentage of identity of the nucleotides was 100%. For statistical significance, Fisher's exact test was used as test of association and a P value ≤ 0.05 was applied as a cut-off for statistical significance.

Variant calling, mapping and *de novo* assembly was done using CLC Assembly Cell Version: 4.4.2.133896 (Qiagen Bioinformatics). Minimum coverage was set to 10× and SNP ratio cut-off for SNP support was set to 90%. Gubbins version 2.3.1, with standard settings, was used to remove SNPs from recombination regions.⁷ Maximum likelihood trees were inferred with FastTree/2.1.8⁸ using GTR nucleotide substitution model and bootstrapping (1000 replicates). Visualization of trees and metadata was done using iTOL (<https://itol.embl.de/>).⁹

The study was ethically approved by AAU-ERB, AHRI-ERC and NERC-Ministry of Science and Technology, Ethiopia. Written consent was obtained from study participants, and the findings for each patient were communicated to the physician in charge of care of each patient.

Results

A total of 1087 patients ($n = 802$ admitted to the hospital and $n = 285$ seeking care at the outpatient department) with suspected infections (pneumonia, diarrhoea, wound infections and urinary tract infections) from four units of the hospital—the medical unit ($n = 509$), the surgical unit ($n = 361$), the paediatric unit ($n = 175$) and the ICU ($n = 45$)—were included in the study. Based on the specific condition of each patient, sputum, wound swab, urine and stool samples were collected. The prevalence of ESBL was higher at the surgical unit (88.8%) and the paediatric unit (86.5%) compared with the ICU (66.7%) and the medical unit (57.7%) (Figure 1).

A total of 146 *K. pneumoniae* strains were isolated and 111 (76%) were ESBL producing. Thirty-three (22.6%) of the ESBL-KP were non-susceptible to carbapenems, but only one strain encoded a carbapenemase gene (bla_{NDM-1}). The bla_{CTX-M} gene was the most prevalent ESBL-type and variants included: $bla_{CTX-M-15}$ (84%, 92/109), $bla_{CTX-M-11}$ (3.6%, 4/109), $bla_{CTX-M-14}$ (1.8%, 2/109), as well as $bla_{CTX-M-3}$ and $bla_{CTX-M-9}$ (each accounting for 0.9%, 1/109). Only one strain carried two bla_{CTX-M} genes ($bla_{CTX-M-14}$ and $bla_{CTX-M-15}$). Multiple variants of the bla_{SHV} -genes were detected, but they were less prevalent than other ESBL genes: bla_{SHV-11} (21.1%, 23/109), bla_{SHV-1} (12.8%, 14/109), bla_{SHV-28} (6.3%, 7/109), bla_{SHV-27} (3.6%, 4/109) and bla_{SHV-26} (2.7%, 3/109). Among other β -lactamase genes, the bla_{TEM} gene bla_{TEM-1b} (69.7%, 76/109) was the most prevalent, the bla_{OXA} , bla_{CMY} , and bla_{TEM-1A} genes were also detected, but with lower prevalence (Table 1).

Phenotypic analysis showed a high prevalence of resistance to non- β -lactam antimicrobials like aminoglycosides [81.8% (90/109) for gentamicin and 5.5% (6/109) for amikacin], ciprofloxacin (63.3%, 69/109), sulfamethoxazole (95.4%, 104/109), and piperacillin/tazobactam (50%). Based on phenotypic analysis, 93% (102/109) of the strains were resistant to three or more classes of

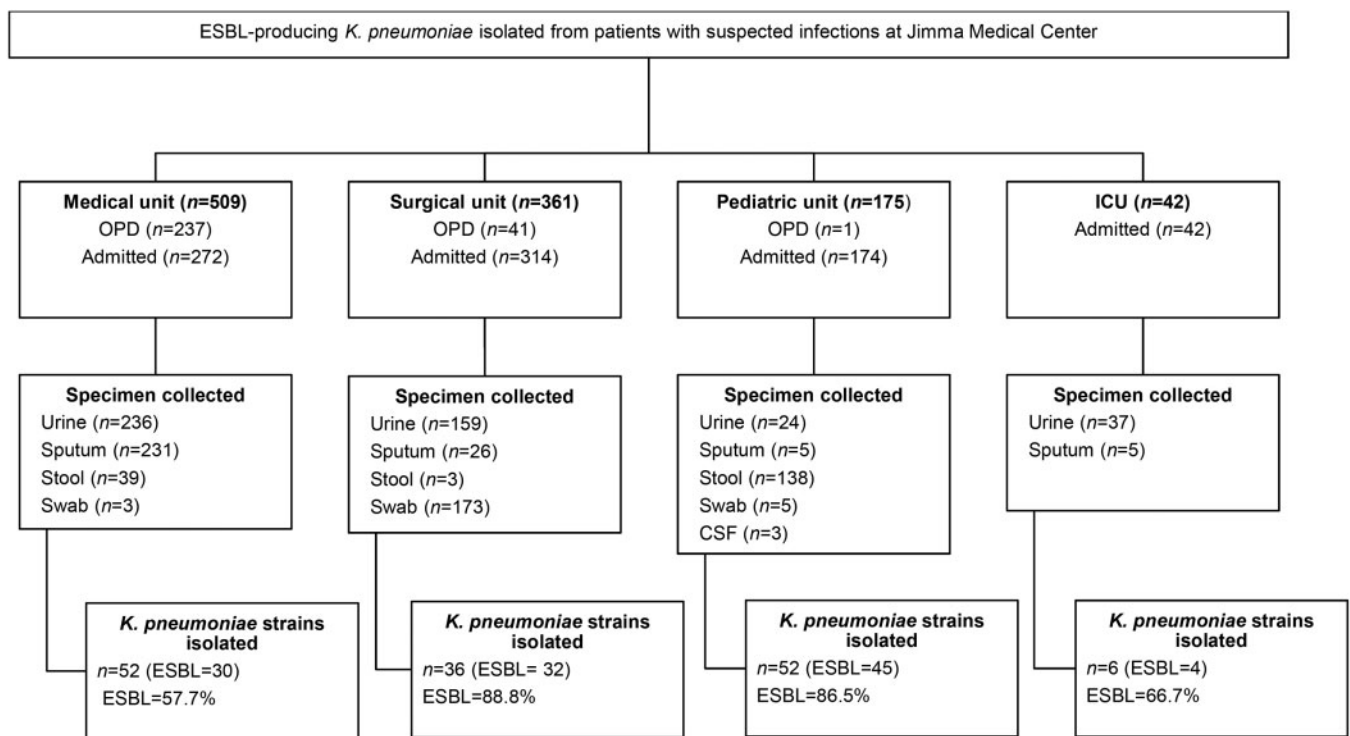


Figure 1. Flow chart showing prevalence of ESBL-KP strains at different units of the hospital. OPD, outpatient department.

antimicrobials, 76% (83/109) were resistant to four or more classes of antimicrobials, and 15% (17/109) were resistant to six classes of antimicrobials (Table 2). Similarly, the prevalence of genetic determinants of resistance to non- β -lactam antimicrobials was high among these ESBL strains. Aminoglycoside resistance genes were present in all ESBL-producing strains, and other commonly encountered resistance gene encoded resistance to fluoroquinolones (72.4%), sulphonamides (84.4%), trimethoprim (61.3%) and phenicols (33.8%). The *aac(6′)-Ib-cr* gene was the most prevalent aminoglycoside and fluoroquinolone resistance gene (65.1%) (Table 1) and 95.7% of the *aac(6′)-Ib-cr* co-existed with *bla_{CTX-M-15}*. The coexistence of *bla_{CTX-M-15}* and *aac(6′)-Ib-cr* genes in the same strain was statistically significant ($P < 0.0001$).

MLST revealed more than 40 different STs. Some of the STs were novel, whereas ST218 (13%, $n = 7$), ST15 (11.1%, $n = 6$), ST17 (11.1%, $n = 6$), ST147 (11.1%, $n = 6$) and ST39 (9.3%, $n = 5$), were most prevalent. ST14, ST15, ST17, ST20, ST101, ST147, ST218 and ST340 were among the epidemic clones detected in this study and overall epidemic clones accounted for 22.0% of the isolates (Table S1, available as Supplementary data at JAC-AMR Online).

At least one plasmid replicon type was identified in 88.9% (97/109) of the strains. IncFIB (IncFIBK, IncFIBMar, IncFIBpQil, 32.9%, 32/97), IncR (24.7%, 24/109) and IncQ1 (12.4%, 12/109) were the most prevalent plasmid replicon types (Table S2).

Genetic determinants of virulence, including capsules, O-LPSs, siderophores and adhesins (*fimH-1* and *fimH-3*), were analysed. KL62 (9.3%, 10/108), KL57 (9.3%, 10/108), KL25 (6.5%, 8/108) and KL64 (6.5%, 8/108) were the most prevalent capsular types. Regulators of mucoid phenotype (*rmpA1/rmpA2*) were detected among epidemiologically important strains. Both Type 1 and Type 3 adhesive fimbrial antigens were encoded by all strains.

The O-LPSs O1 (53.7%, 57/108) and O2 (23.15%, 25/108) were the most prevalent types (Table S3). Several genetic determinants of siderophores were identified, the prevalence varied from 6.5% to 42.5%. Yersiniabactin *irp1* and *irp2* (6.5%, 7/108), and *ybtQ* (42.6%, 46/108), salmochelin *iroBCDE* (7.4%, 8/108), and aerobactin *iucABCD/iutA* (9.2%, 10/108) were the most prevalent siderophores (Table S4).

An SNP-based maximum likelihood tree showed several clusters of clonally related high-risk clones that were isolated from different units in the hospital (Figure 2). Multiple clonally related strains of ST15, ST17, ST39, ST48, ST147 and ST218, were isolated from clinical samples collected from surgical, paediatric, medical and intensive care units. The findings demonstrate nosocomial transmission of *K. pneumoniae* featuring MDR and hypervirulence.

Discussion

The very high prevalence of ESBL-KP in this study is higher than a number of previous reports from Africa (Tanzania, South Africa, Gabon, Guinea-Bissau, and many other parts of the continent).^{10,11} However, it might be comparable to the finding of a study conducted in 2014 in Bamako, Mali, which reported 76.9% among *K. pneumoniae*.¹² The fact that 93% of the ESBL-KP strains were resistant to at least three classes of antimicrobials and 15% to at least six classes of antimicrobials may indicate the tip of the iceberg of a high prevalence of MDR *K. pneumoniae* strains disseminating in hospitals in Ethiopia. In most African countries, such evidence might have been obscured because of the lack of sufficient technically standardized studies. Similarly, a recent review on AMR from Africa reported that there is a lack of updated AMR data for 40% of the African countries.¹³ The same review noted that the

Table 1. List of AMR genes and their prevalence in *K. pneumoniae* strains isolated at Jimma Medical Center, Ethiopia

Serial no.	Gene description and resistance mechanism				Genes detected	Prevalence
	Class of antimicrobial	sub-class genes	putative mechanisms	drugs targeted		
1	Aminoglycosides	acetylation genes	acetyl transferase	aminoglycosides	<i>sac (3)-IIa</i>	18 (16.5%)
			acetyl transferase	aminoglycosides	<i>sac (3)-IIa</i>	1 (0.9%)
			acetyl transferase	aminoglycosides and fluoroquinolones	<i>sac (6) Ib-cr</i>	71 (65.1%)
		adenylation	acetyl transferase	aminoglycosides	<i>sac (6)-IIc</i>	2 (1.8%)
			acetyl transferase	aminoglycosides	<i>sacA4</i>	2 (1.8%)
			adenyl transferase	aminoglycosides	<i>sacA1</i>	4 (3.6%)
			tetracycline efflux	aminoglycosides	<i>sacA2</i>	14 (12.8%)
			adenyl transferase	aminoglycosides	<i>sacA5</i>	1 (0.9%)
		streptomycin	adenyl transferase	aminoglycosides	<i>sacB</i>	3 (2.7%)
			streptomycin phosphotransferase	aminoglycosides	<i>strA</i>	45 (41.3%)
		2	β -lactams	phosphorylation	streptomycin phosphotransferase	aminoglycosides
streptomycin phosphotransferase	aminoglycosides				<i>aph (3')-Ia</i>	10 (9.2%)
β -lactamase	phosphotransferase			cephamycins	<i>bla_{CMY-6}</i>	1 (0.9%)
	β -lactamase			cephamycins	<i>bla_{CMY-42}</i>	2 (1.8%)
	β -lactamase			aminopenicillins and cephalosporins	<i>bla_{CTX-M-11}</i>	4 (3.6%)
ESBL	ESBL			aminopenicillins and cephalosporins	<i>bla_{CTX-M-14}</i>	2 (1.8%)
	ESBL			aminopenicillins and cephalosporins	<i>bla_{CTX-M-15}</i>	92 (84.4%)
	ESBL			aminopenicillins and cephalosporins	<i>bla_{CTX-M-3}</i>	1 (0.9%)
	ESBL			aminopenicillins and cephalosporins	<i>bla_{CTX-M-9}</i>	1 (0.9%)
β -lactamase	β -lactamase			aminopenicillins	<i>bla_{OXA-1}</i>	38 (34.8%)
	ESBL	aminopenicillins and cephalosporins	<i>bla_{OXA-10}</i>	12 (11.0%)		
β -lactamase	β -lactamase	aminopenicillins	<i>bla_{TEM-1B}</i>	76 (69.7%)		
	β -lactamase	aminopenicillins	<i>bla_{TEM-1A}</i>	2 (1.8%)		
	β -lactamase	aminopenicillins	<i>bla_{SHV-1}</i>	14 (12.8%)		
β -lactamase	β -lactamase	aminopenicillins	<i>bla_{SHV-11}</i>	23 (21.1%)		
	β -lactamase	aminopenicillins	<i>bla_{SHV-26}</i>	3 (2.7%)		
	ESBL	aminopenicillins and cephalosporins	<i>bla_{SHV-27}</i>	4 (3.6%)		
	β -lactamase	aminopenicillins	<i>bla_{SHV-28}</i>	7 (6.3%)		
3	Carbapenem	carbapenemase	aminopenicillins and cephalosporins	<i>bla_{NDM-1}</i>	1 (0.9%)	
			aminopenicillins, cephalosporins and carbapenems	<i>catB3</i>	31 (28.4%)	
4	Phenicol	acetyl transferase	chloramphenicol	<i>catB4</i>	6 (5.4%)	
			chloramphenicol	<i>catB4</i>	6 (5.4%)	
5	Trimethoprim	dihydrofolate reductase	sulfamethoxazole/co-trimoxazole	<i>dfrA1</i>	5 (4.5%)	
			sulfamethoxazole/co-trimoxazole	<i>dfrA12</i>	10 (9.2%)	
		dihydrofolate reductase	sulfamethoxazole/co-trimoxazole	<i>dfrA17</i>	2 (1.8%)	
		dihydrofolate reductase	sulfamethoxazole/co-trimoxazole	<i>dfrA27</i>	35 (32.1%)	
		dihydrofolate reductase	sulfamethoxazole/co-trimoxazole	<i>dfrA5</i>	1 (0.9%)	
		dihydrofolate reductase	sulfamethoxazole/co-trimoxazole	<i>dfrA7</i>	14 (12.8%)	

Table 2. Prevalence of MDR, type of antimicrobial and the combined resistance of the *K. pneumoniae* strains

Strains, n (%)	Antibiogram of ESBL strains of <i>K. pneumoniae</i> (%)	Number of drugs	Number of antimicrobial classes
8 (7%)	CTX-CAZ-SXT (62.5%) CTX-CAZ-GEN (12.5%) CTX-CAZ-GEN (25%)	3	2
19 (17%)	CTX-CAZ-SXT-GEN (57.9%) CTX-CAZ-CIP-SXT (26.3%) CTX-CAZ-MEM-GEN (5.2%) CTX-CAZ-SXT-MEM-GEN (10.5%)	4	3
40 (36%)	CTX-CIP-SXT-GEN (2.5%) CTX-CAZ-CIP-SXT-GEN (35%) CTX-CAZ-TZP-SXT-GEN (32.5%) CTX-CAZ-CIP-SXT-MEM (12.5%) CTX-CAZ-SXT-MEM-GEN (10%) CTX-CAZ-TZP-CIP-SXT (5%) CTX-CAZ-TZP-CIP-AMK (2.5%) CTX-TZP-CIP-SXT-GEN (3.8%)	5 4 5	4
26 (24%)	CTX-CAZ-TZP-CIP-SXT-GEN (73%) CTX-CAZ-CIP-SXT-MEM-GEN (19.2%) CTX-CAZ-TZP-SXT-MEM-GEN (3.8%)	5 6	5
17 (16%)	CTX-CAZ-TZP-CIP-SXT-MEM-GEN (94.2%) CTX-CAZ-TZP-CIP-SXT-ETP-MEM-GEN-AMK (5.8%)	7 9	6

CTX, cefotaxime; TZP, piperacillin/tazobactam; CIP, ciprofloxacin, CAZ, ceftazidime, AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; ETP, ertapenem; MEM, meropenem; IPM, imipenem; GEN, gentamicin.
Bold indicates the most prevalent resistance patterns.

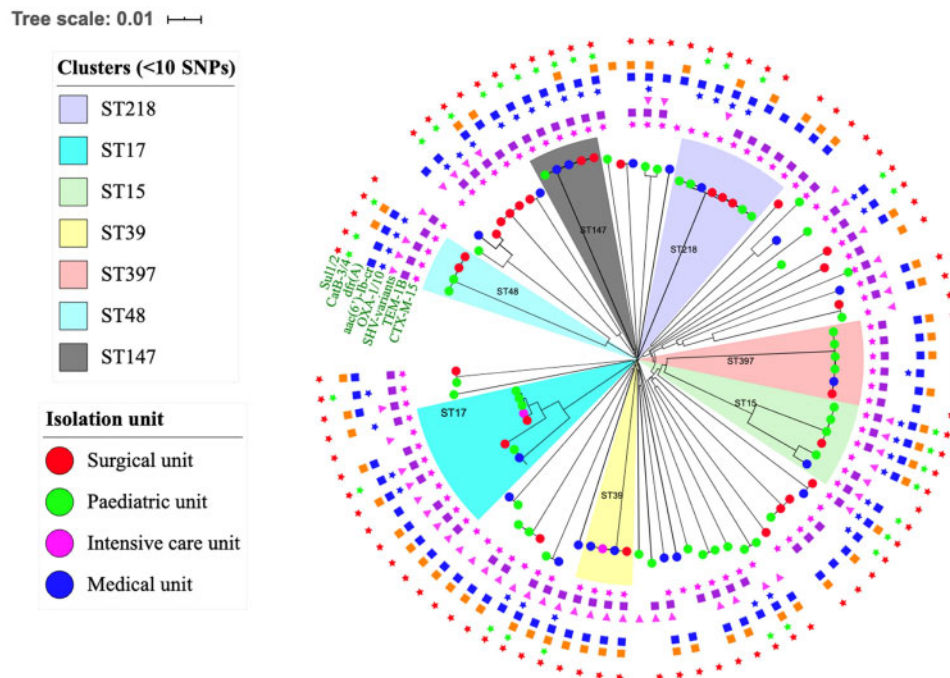


Figure 2. Clusters of epidemic clones isolated from the surgical unit, paediatric unit, ICU and medical unit of the hospital.

yersiniabactin, salmochelin, aerobactin), fimbriae adhesins (Fim-1, and Fim-3) and miscellaneous (Table S4). Among these virulence genes, the regulators of mucoid phenotype genes (*rmpA* and *rmpA2*) for hypermucoviscous property of strains were identified exclusively with KL57, one of the most prevalent capsular types, and the KL57 was predominantly detected in ST218 strains. Besides the hypercapsular phenotype, these clones encoded multiple groups of siderophores (molecules for scavenging iron) (*iroBCDN*, *iucABCD*, *iutA*, *entA*, *ybtA*), and FimH-3 (*mrkABCD* clusters). On the other hand, an experimental study from Russia characterized strains with similar genotype (*K. pneumoniae*-ST218-KL57) as hypermucoviscous/hypervirulent clones.²³ Other epidemiological studies from Russia and China showed that KL57 capsular types were associated with diarrhoea, pyogenic liver abscess, meningitis and bloodstream infections.^{24,25}

With regard to LPS structures, the O2V2 types were predominant among the strains of ST218-KL57 clone. As described earlier, all of them encoded KL-57 capsular type, the similar patterns of surface molecules (KL-locus and O-LPSs) on the surface of high-risk and hypervirulent *K. pneumoniae* strain might be important information for intervention strategies targeting surface molecules. The O-LPSs were rarely reported from epidemiological studies, however we reported here the unique pattern of KL57-O2V2 surface molecules identified on a high-risk clone (ST218), which might be important epidemiological knowledge. However, large scale studies are required to consolidate evidence for prevention strategies to target these structures.

Furthermore, analysis of a population structure of the strains showed nosocomial transmission between the different units of the hospital. Seven independent outbreak clusters of high-risk clones were detected by a phylogenetic tree constructed using SNP analysis of the shared core genome. A clonal cluster of strains isolated from different clinical samples collected from surgical, paediatric, intensive care and medical units of the hospital indicates a likely occurrence of transmission clusters. Most importantly, the detection of multiple nosocomial transmission clusters of high-risk clones of *K. pneumoniae* can severely compromise the standard of care and patient safety at this hospital, where therapeutic options are limited.

The high prevalence and nosocomial transmission of multiple ESBL-producing high-risk clones of *K. pneumoniae* strains from a low-income country is a severe problem. In a setting where the microbiology laboratory capacity is compromised, supply of antimicrobials is limited and there is a lack of personnel with specialty training to consult prescribers, patient care and safety can be significantly affected. Moreover, lack of stringent infection control practices and functional antimicrobial stewardship programme may warrant further spread of more stringent high-risk clones.

Conclusions

The prevalence of ESBL-KP was very high and *bla*_{CTX-M-15} was the predominant ESBL gene. The spread of resistance genes was mediated by multiple high-risk clones that also encoded epidemic plasmids. The prevalence of carbapenemases was low, however, the detection of *bla*_{NDM-1} carbapenemase in a hypervirulent high-risk clone is a worrisome challenge for the future. We were able to

document nosocomial transmission of multiple high-risk clones between different units in the hospital.

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Transparency declarations

None to declare.

Author contributions

T.S. contributed to the design and conception of the study, data collection, data analysis and interpretation, and writing the manuscript. C.G.G. contributed to the design and conception of the study, data acquisition and analysis, and writing the manuscript. D.A. and Y.W. contributed to the design and conception of the study and revision of the manuscript. S.N. contributed to data analysis and drafting and revision of the manuscript. F.W. contributed to the study design and revision of the manuscript. A.A. contributed to the study design, data acquisition and revision of the manuscript. All authors read and approved the manuscript for submission.

Supplementary data

Tables S1 to S6 are available as [Supplementary data](#) at JAC-AMR Online.

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