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Multi-clonal spread of *Klebsiella pneumoniae* across hospitals in Khartoum, Sudan

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Abstract

Introduction and Objectives: Multidrug resistant (MDR) *Klebsiella pneumoniae* is increasing worldwide with poorly characterized epidemiology in many parts of the world, particularly in Africa. This study aimed to investigate the molecular epidemiology of *K. pneumoniae,* to identify the diversity of Sequence Types (ST) and to detect carbapenem resistance genes in major regional hospitals in Khartoum, Sudan,

Methods: *K. pneumoniea* isolates (n=117) were cultured from four hospitals in Khartoum, from April 2015 to October 2016. The isolates were characterised by sequencing of 16S-23S rDNA internal transcribed spacer (ITS) region. Molecular epidemiology was determined by Multilocus sequence typing (MLST), and analysed by maximum likelihood phylogeny (PhyML). Antimicrobial Susceptibility was determined by disk diffusion. Isolates phenotypically resistant to carbapenem were screened for carbapenemase genes: bla_{NDM}, bla_{OXA48}, bla_{IMP}, bla_{VIM} and bla_{GES} by PCR.

Results: ITS sequencing confirmed the 117 isolates as *K. pneumoniae.* MLST revealed 52 different STs grouped in 4 distinct clusters by PhyML. All isolates were MDR and carbapenemase-producing *K. pneumoniae* (CP-KP) isolates accounted for 44/117 (37.6%) mostly harbouring bla_{NDM} (28/44) and $bla_{\text{OXA-48}}$ (7/44) with several isolates harbouring multiple genes.

Conclusion: MDR and CP-KP *K. pneumoniea* is widespread in Khartoum hospitals, with a diverse population of 52 ST clustering in 4 major lineages. There is an urgent need for systematic epidemiological studies of drug-resistant infections across all healthcare institutions in Sudan to inform local infection prevention and control strategies.

Keywords: *K. pneumoniae*, Sudan, epidemiology, MLST

1 Introduction

The incidence of multi-drug resistant (MDR) and carbapenemase-producing *Klebsiella pneumoniae* (CP-KP) infections has increased during the last decade throughout the world, assigning *K. pnuemoniae* as one of the global priority pathogens by the World Health Organisation (WHO) (1,2) with great medical significance implicated in urinary tract infections, pneumonia, bacteraemia, meningitis, and abscesses (3). Therefore, MDR *K. pneumoniae* is considered a significant health problem associated with significant morbidity and mortality due to limited antibiotic treatment options (4). Carbapenems are usually the last-resort antibiotic by virtue of their broad spectrum of activity, however CP-KP has created significant clinical challenges for clinicians globally, resulting in ineffective treatments and high rates of clinical failure. Furthermore, this problem is aggravated by the localisation of resistance genes on mobile elements facilitating gene transfer to susceptible isolates (5).

The burden of MDR is underestimated in many low- and middle-income countries (LMICs), due to limitations in diagnostic facilities (6). In Sudan, epidemiology of MDR and CP-KP is not well described despite being a major public health threat, with fragmented data on the prevalence and distribution (7–9). Quantification of CP-KP in healthcare facilities and understanding the evolving epidemiology of CP-KP in Sudan is critical to informing national and regional infection prevention and control (IPC) efforts.

We have previously reported a high rate of misidentification of *K. pneumoniae* across Khartoum hospitals (10). In this study, we aimed to characterise the local epidemiology by multi-locus sequence typing (MLST), the prevalence of carbapenemase genes among *K. pneumoniae* isolates across Khartoum State hospitals, thereby determining the level of inter/intra-hospital transmission.

2 Material and Methods:

2.1 Bacterial isolates and antimicrobial susceptibility testing:

A total of 117 isolates of *K. pneumoniae* from patient samples were cultured in clinical microbiology laboratories (CML) of four major teaching hospitals in Khartoum: Ribat National Hospital (RNH), Omdurman Teaching Hospital (OTH), Soba University Hospital (SUH) and Khartoum Bahri Teaching Hospital (KBTH) between April 2015 and October 2016. CML identify clinical specimens to the genus levels by conventional methods phenotypic and biochemical methods. No specific selection criteria was implemented, as the study aimed to collect and characterise all *K. pnuemoniae* isolates identified in the hospitals' CML. All acquired isolates were then confirmed genotypically by amplification of 16S-23S rDNA internal transcribed spacer (ITS) of *K. pneumoniae* as described by our preceding study (11). All primer sequences and expected amplicon sizes are listed in Table S1. Clinical data associated with the isolates were collected which include: age, gender, location of patient, type of culture, and antibiotic therapy before and during the infection.

Susceptibility tests were done by disk diffusion for the following 10 antibiotics: Amoxicillin/Ampicillin (AMC/AMP) (30µg); Piperacillin-Tazobactam (TPZ) (110µg); Cefoxitin (FOX)(30µg); Ciprofloxacin (CIP)(5µg); Gentamicin (CEN)(10µg); Amikacin (AK)(30µg); Trimethoprim-Sulfamethoxazole (SXT)(30µg); Meropenem (MEM)(10µg); Imipenem (IMP)(10µg). The procedure was performed and results interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (12).

2.2 Molecular typing of *K. pneumoniae* isolates:

MLST was done to identify the STs of all the *K. pneumoniae* isolates by amplification and sequencing of the 7 housekeeping genes (13). The results of sequences were analyzed in the

PubMLST database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) and BLAST (https://blast.ncbi.nlm.nih.gov/), then assigned an allele number. The allele numbers are combined to yield a specific ST.

A concatenated alignment with maximum likelihood phylogeny (PhyML) was constructed using Seaview to determine relatedness of isolates (14), and the PhyML was analysed with metadata using Phandango (15).

2.3 Molecular detection of carbapenemase genes:

All isolates phenotypically resistant to carbapenems were screened for the presence of carbapenemase genes: *bla*$_{NDM}$, and *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{OXA48}$ and *bla*$_{GES}$ by multiplex-PCR using specific primers listed in Table S1 (17) . PCR reaction conditions were prepared by using ready master mix (APSLABS, India), 0.5µl of each primer (25nmole concentration) and 1 µl of template DNA (10 ng) in a total volume of 25µl. PCR thermal profile for NDM comprised of initial denaturation at 94C° for 10 min followed by 30 cycles of 1 min at 94C°, 1 min at 60C° and 1 min at 72C°, and final extension step of 10 min at 72C°. The PCR amplification for multiplex (VIM, IMP and KPC) was carried out as follows: 94°C for 10 min; 30 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 1 min; and 72°C for 7 min. The same conditions were used for multiplex GES and OXA-48 PCR, but with the annealing temperature at 57C° instead of 55C°. The PCR products were analyzed by gel electrophoresis.

3- Results:

A total of 117 MDR *K. pneumoniae* isolates were cultured from different clinical samples collected from in- and out-patients across four hospitals in Khartoum, Sudan. Table 1 summarizes the

distribution pattern of *K. pneumoniae* by age groups, gender, hospital, and source of specimens. The highest number of *K. pneumoniea* isolates were from Ribat National Hospital (RNH; n=41; 35.0%), followed by Omdurman Teaching Hospital (OTH; n=34; 29.1%), Soba University Hospital (SUH; n=27; 23.1%) and Khartoum Bahri Teaching Hospital (KBTH; n=15; 12.8%). Isolates were obtained from a range of ages, however the largest number of isolates were from infants (<1 year, 23.9%). More than 80% of isolates were from inpatients, from a variety of samples, but more commonly blood and urine samples or wound swabs (70.1% collectively).

As listed in Table 2, all isolates were MDR, with particularly high resistance rates (>50%) to Amoxicillin/Ampicillin, Piperacillin/tazobactam, Ciprofloxacin and Gentamicin. The majority of isolates were sensitive (>60%) to Amikacin and Trimethoprim/Sulfamethoxazole. Carbapenem resistance was observed in 50 isolates (42.8%), with total resistance at 13.7% and 29.1% for Imipenem and Meropenem, respectively. Antibiotic use prior to the first positive culture was common (61.4%) in patients with a carbapenem resistant *K. pneumoniae*; only 17/44 (38.6%) had no antibiotic therapy in the preceding 14 days (Table S2).

MLST revealed 52 different STs, 15 of which are novel and assigned to ST3460-3474. STs 101, 383, and 649 were present in different hospitals in Khartoum as seen in Table S3. The STs of the 44 CP-KP revealed that recurrent STs were ST 383 (n=8, in SUH, RNH, OTH), followed by ST 101 (n=5, in OTH, KBTH, RNH), ST 48 (n=3 in KBTH), 649 (n=3, SUH &RNH), ST 846 (n=3, RNH) and 3229 (n=2, KBTH), all other STs were identified in individual isolates (Table 3).

As seen in Figure 1, there is a large diversity of STs among the isolates with several lineages present. Results indicate that despite distinct STs present in each hospital, with only a few recurrent STs listed above, all STs fall into 4 main clusters that are present across hospitals. Notably, ST2461 and ST2674 are highly similar (part of cluster 2 in Figure 1) however isolates come from RNH and SUH,

indicating inter-hospital spread. Cluster 4 (highlighted in green) appears to contain the largest diversity: with 3 sub-clusters across the 4 hospitals of isolates that are both CP-KPs and non-CP-KP. Some hospitals appear to have clonally related strains, such as in OTH: ST-654, -1298, -657, -2870, -219, -2260, -524, -2461, -2674, belonging to cluster 2 (Figure 1). On the other hand, SUH appears to have a pool of diverse CP-KP and non-CP-KP. Notably, the novel STs 3460-3474 are all carbapenem sensitive with the exception of ST 3467.

All isolates exhibiting phenotypic resistance to carbapenems were screened for acquired carbapenemases as outlined in the methods section. Fourty-four isolates (37.6% of the total 117 isolates) harboured NDM (n=32), OXA-48 (n=10), GES (n=6) and VIM (3), of which several isolates co-harboured several genes: 2 with NDM & VIM, 2 with OXA-48 & GES, 1 NDM & GES, and 1 with NDM, VIM and OXA-48 (Figure S4). NDM is present across a range of STs in multiple hospitals.

4- Discussion:

Khartoum city is the capital of Sudan and includes three localities: Khartoum, Bahri and Umdurman. Hospitals in the three localities are under the umbrella of Khartoum State Ministry of Health and they provide service to the residence of the State as well as those who are referred from other states. The study aimed to characterize the molecular epidemiology of a total of 117 MDR *K. pneumoniae* across 4 major teaching hospitals who serve the majority of the residence of the city: RNH and SUH are located in Khartoum locality, OTH in Omdurman locality, and KBTH located in Bahri locality. The majority of isolates were from RNH, and as detailed in Table 1, the isolates were most commonly from infants (<1 year old). Carbapenem resistance was unexpectedly high (>40%), while >60% of isolates were sensitive to Amikacin. Our data (Table S2) revealed that antibiotic usage (14 days

151 before and during hospital admission) was a risk factors for CP-KP mostly in patients with combined
152 antibiotic therapy.

153 During the 19-month surveillance period 37.6% (n=44/117) of *K. pneumoniae* isolates harbored a
154 carbapenemase, namely NDM, VIM, OXA-48 and GES. Patients who were colonized or infected
155 with CP-KP have high rates of certain comorbidities including renal disease, diabetic wounds, and
156 solid malignancy (data not shown). Notably, the majority of CP-KP isolates were isolated from urine
157 and wound were the predominant source, followed by blood, sputum and pus samples (Table 1),
158 similar to the results of other studies in Sudan, Uganda Nigeria, (20–22).

159 The emergence of CP-KP limits the use of carbapenems in patients with severe infections, leading to
160 increased mortality rates. In the last decade, infections caused by carbapenem resistant *K.*
161 *pneumoniae* increased significantly in many countries in the region (Africa and the Middle East) (20,
162 23–28). Currently in Sudan, carbapenems are the only available choice for the treatment of MDR *K.*
163 *pneumoniae* and other ESBL and AmpC-producing Gram-Negatives. However in most cases,
164 antibiotic prescription of carbapenems is not based on AST, but rather based on previous treatment
165 failures or as prophylaxis. There is therefore an urgent need to address improving CML diagnostics
166 which in turn would have a direct impact on limiting the misuse of antibiotics, particularly
167 carbapenems. It is important to note that the limited diagnostic capacities in CML across hospitals in
168 Khartoum has led to frequent outsourcing of cultures and general laboratory diagnostics where
169 patients are in many instances directed to private laboratories. Subsequently, the local data on
170 epidemiology and resistance is fragmented.

171 In this study, the prevalence of MDR was 51.2% among the *K. pneumoniae* isolates, with >40% of
172 isolates being carbapenem resistant. Our results indicate that isolates remain >60% sensitive to
173 Amikacin and Trimethoprim-Sulfamethaxazole (SXT), however Gentamicin resistance reached 64%.

Most antibiotics in Sudan are obtainable over the counter, which may have contributed to the high MDR rates noted in the study. Antibiotic usage in the community and its role in the transmission of resistance is an important issue to study in the future.

As seen in Table 1, CP-KP was isolated from in-patients in 36/44 (81.8%), and in 8/44 (18.2%) among out-patients, indicating the possible dissemination of CP-KP in community-acquired infections. Carbapenem resistance mediated by NDM was present across a diversity of STs and lineages, suggesting the successful dissemination and maintenance among diverse strains.

The molecular epidemiological characterization of *K. pneumoniae* in Khartoum, Sudan revealed a high diversity of 52 different STs, including 15 novel STs. However the PhyML analysis revealed that despite this diversity in STs, they fall within 4 main clusters circulating in different hospitals. The largest diversity of STs was observed in SUH & RNH (Figure 1), where it is apparent that multiple lineages are present simultaneously in the hospital. OTH, on the other hand, has the least diversity and appears to have one major CP-KP clone of closely related STs (645, 1298, 657, 2870, 219, and 2260) in addition to other sporadic STs. The diversity observed in this study may also represent isolates brought to the hospitals from diverse geographical settings, as these hospitals are tertiary hospitals serving most of the country.

Successful global clones ST-383 and -101 were identified in our study and are present in multiple hospitals indicating the introduction and maintenance of international clones in Sudanese hospitals, thereby confirming the role of travel and immigration in transmission of resistance worldwide (18,19). It is also important to note that despite the global spread of ST-258-KPC-producer, in our study we did not detect the KPC gene, and the global ST258 was not identified (4).

5- Conclusion:

Our study provides an overview of the molecular epidemiology of *K. pneumonaie* in Khartoum thereby collecting samples from 4 hospitals representing the 3 regions, revealing a heterogeneous population of MDR *K. pneumoniae* isolates and the presence of 4 major clusters highlighting a large inter-hospital spread of diverse clones in Khartoum, Sudan. The data highlighted an alarming rate of ~40% carbapenem resistance.

Table 1: Distribution pattern of *K. pneumoniae* and carbapenemase producing K. *pneumoniae* by age groups, gender and source of specimens:

Variables	*K. pneumoniae* (n=117) N (%)	CP-KP (n=44) N (%)
Age/ years		
<1year	28 (23.9%)	9 (32.1%)
1-15	21 (17.9%)	9 (42.9%)
16-30	17 (14.5%)	8 (47.1%)
31-45	16 (13.7%)	6 (37.5%)
46-60	19 (16.3%)	6 (31.6%)
>61	16 (13.7%)	6 (37.5%)
Gender		
Male	44 (37.6%)	16 (36.4%)
Female	73 (62.4%)	28 (38.4%)
Hospitals		
Reibat National Hospital (RNH)	41 (35.0%)	16 (36.4%)
Omdurman Teaching Hospital (OTH)	34 (29.1%)	13 (29.5%)
Soba University Hospital (SUH)	27 (23.1%)	8 (18.2%)
Khartoum Bahery Teaching Hospital (KBTH)	15 (12.8%)	7 (15.9%)
Hospital status		
Inpatient	103 (88.0%)	36 (81.8%)
Outpatient	14 (12.0%)	8 (18.2%)
Source of specimens		
Blood	28 (23.9%)	8 (18.2%)
Urine	27 (23.1)	13 (29.5%)
wound swab	27 (23.1%)	13 (29.5%)
Umbilical swab	11 (9.4%)	5(11.4%)
Sputum	9 (7.7%)	1 (2.3%)
Pus	8 (6.83%)	1 (2.3%)
Nasal swab	4 (3.42%)	3 (6.8%)
C.S.F	2 (1.7%	0 (0%)
Eye swab	1 (0.85%)	0 (0%)

Table 2: Antimicrobial susceptibilities profiles of *K. pneumoniae*

Antibiotic	*K. pneumoniae* (n=117)		
	Susceptible	Intermediate	Resistant
	N (%)	N (%)	N (%)
AMC/AMP	2 (1.7%)	0 (0%)	115 (98.3%)
TZP	7 (5.9%)	0 (0%)	110 (94.0%)
Cefoxitin	7 (5.9%)	74 (63.2%)	36 (30.8%)
Ciprofloxacin	45 (38.5%)	12 (10.3%	60 (51.3%)
Impenem	89 (76.1%)	12 (10.3%)	16 (13.7%)
Meropenem	79 (67.5%)	4 (3.4%)	34 (29.1%)
Gentamycin	41 (35.0%)	1 (0.85%)	75 (64.1%)
Amikacin	78 (66.5%)	2 (1.7%)	37 (31.6%)
SXT	79 (67.5%)	1 (0.85%)	37 (31.6%)

TZP: piperacillin-tazobactam; SXT: trimethoprim-sulfamethoxazole; AMC/AMP: amoxicillin/ampicillin.

211 **Table 3: Distribution of identified and assigned STs for CP-KP isolates by MLST:**

ST	Frequency (n=)	Carbapenemase		Hospital
24	1 CP-KP	NDM	1 (2.3%)	OTH
48	3 CP-KP	NDM	3 (6.8%)	KBTH
101	5 CP-KP	NDM	5 (11.4%)	All except SUH
119	1 CP-KP	NDM	1 (2.3%)	STH
219	1 CP-KP	NDM	1 (2.3%)	OTH
376	1 CP-KP	GES	1 (2.3%)	SUH
383	8 CP-KP	2-NDM, 1-GES, 3-OXA-48, 1-NDM+GES, 1-OXA-48+GES	8 (18.2%)	All except KBTH
437	1 CP-KP	NDM	1 (2.3%)	RNH
462		OXA-48	1 (2.3%)	RNH
524		NDM	1 (2.3%)	SUH
589		NDM	1 (2.3%)	RNH
649	3 CP-KP	2-NDM, 1-OXA-48	3 (6.8%)	OTH&RNH
654		OXA-48	1(2.3%)	OTH
657		NDM	1 (2.3%)	OTH
677		NDM	1 (2.3%)	RNH
846	2 CP-KP	NDM	2 (4.5%)	RNH
1029		OXA-48	1 (2.3%)	SUH
1289		GES	1 (2.3%)	OTH
1806		NDM+VIM	1 (2.3%)	OTH
2260		NDM	1 (2.3%)	OTH
2461		OXA-48+GES	1 (2.3%)	SUH
2674		NDM+VIM	1 (2.3%)	RNH
2695		NDM	1 (2.3%)	SUH
2870		NDM	1 (2.3%)	OTH
2923		NDM	1 (2.3%)	RNH
3229	2 CP-KP	NDM	2 (4.5%)	KBTH
3467		NDM+ OXA-48+ VIM	1 (2.3%)	SUH

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Figure 1: Maximum Likelihood Phylogeny (PhyML) of concatenated STs identified in the study.

The STs fall into 4 major clusters that are heterogeneous with multiple STs from different hospitals, indicating unique diverse STs to each hospital, but intra-hospital circulating clones. The largest heterogeneity is observed in cluster 4 which appears to have 3 sub-clusters. Two singletons: ST48 and ST2923 are also identified that do not fall within the clusters.

Resistance genes are spread across different STs and clusters (also detailed in Table 3).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All authors contributed equally and participated in design and implementation, analysis, interpretation of the study, and the development of the manuscript. EA collected the strains and conducted the laboratory work and data analysis. LAH performed part of the data analysis. EA and LAH drafted the manuscript. NAE facilitated the isolate collection and reviewed the manuscript. MM supervised the laboratory work and revised the manuscript. All authors had full access to the data and approved the final manuscript.

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Ethical Approval

This work contains no human or animal data. Institutional approval was obtained from The Sudanese Ministry of Health**.**

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