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Molecular characterization of multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli* harbouring extended spectrum beta-lactamases and carbapenemases genes at a tertiary hospital, Kenya

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Summary

Background. Multidrug-resistant (MDR) Gram negative rods are increasingly being reported in sub-Saharan Africa. Molecular investigations play an important role, alongside other measures, in controlling nosocomial infections attributed to these organisms.

This study aimed to determine the common extended spectrum beta-lactamases (ESBL) and carbapenemases genes, and clonal relationship in MDR *Klebsiella pneumoniae* and *Escherichia coli*.

Methods. Fifty-four MDR isolates collected at the Aga Khan University hospital, Nairobi in the month of August 2012 formed the study. These were picked after an increase in the number of resistant strains during the said period was experienced.

Results. *bla*_{CTXM} was present in 41 (74%) of the isolates, while *bla*_{SHV} was detected in 18 (33%) and *bla*_{TEM} in 13 (24%) of the isolates.

Nine (16.7%) of the isolates harboured all three ESBL genes and 8 (14.8%) harboured two. Eight of the isolates (all *E. coli*) had none of the ESBL genes tested. Two isolates harboured carbapenemases genotypes: one had *bla*_{NDM-1} and the other *bla*_{SPM}.

Sequencing matched CTXM-15 and TEM-1 genes in all the isolates harbouring *bla*_{CTXM} and *bla*_{TEM} respectively. However, there was diversity in *bla*_{SHV} with SHV-11 and SHV-12 genes predominant. The isolates were non-clonal.

Conclusions. The isolates mostly harboured *bla*_{CTXM-15} while only a few had carbapenemases genes. Lack of clonality suggests these were the stable circulating strains at the time of the study.

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Contributions: DM conceived and designed the study, performed molecular studies and wrote the manuscript; GR processed the clinical specimens and carried out antimicrobial testing; ACW performed molecular studies and analysed the findings; GR and ACW reviewed and edited the manuscript.

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Introduction

The prevalence of antimicrobial resistance varies greatly between and within countries for different pathogens. Infections with multidrug-resistant (MDR) organisms have been linked with poorer clinical outcomes and prolonged hospitalization on average compared to infections with susceptible pathogens. This is likely exacerbated in resource-limited settings, where access to newer (usually more expensive) antibiotics is often limited by cost (27).

The factors that contribute to the emergence and spread of MDR microorganisms in developing countries are varied. These factors include inappropriate antimicrobial use by both medical personnel and the public, which is due to lack of prescribing skills or lack of access to microbiology laboratory reports (by the medical personnel), or due to unregulated access of patients to antibiotics in the retail market (28). Infection control practices within hospitals are in most places makeshift and often health care personnel may be unaware of emerging or existing multiresistant microorganisms due to lack of surveillance data coupled with inadequate microbiology laboratory facilities (15).

Extended spectrum beta-lactams and carbapenems are among the popular antimicrobials prescribed in severe infections. Therefore, antimicrobial resistance to these agents is of clinical

and public health importance. ESBLs are beta lactamases capable of conferring bacterial resistance to the penicillins as well as first-, second (but not the cephamycins) -, and third-generation cephalosporins, and aztreonam. On the other hand, carbapenemases are carbapenem-hydrolyzing beta-lactamases that confer resistance to a broad spectrum of beta-lactam substrates, including carbapenems. Recent studies performed in the region of East Africa show a wide variation in ESBLs prevalence in hospitals between the different countries with a pooled proportion of 40% reported in a meta-analysis (23). Carbapenemases have been documented in several countries in Africa and Rendani *et al.* in their systematic review have suggested that the problem might be bigger than what is currently estimated due to underreporting (11).

Molecular studies can be helpful in understanding the epidemiology of evolving and established MDR isolates in hospitals. Application of molecular techniques in infection control is gaining worldwide acceptance as the costs of carrying out the tests decrease; an example being whole genome sequencing which was employed to track an outbreak of a carbapenemase producing *Klebsiella pneumoniae* at a hospital in the USA (22). The Aga Khan University Hospital Nairobi (AKUHN) is a 250 bed tertiary and teaching hospital that caters mainly to the middle income population within the city, but also processes samples from across the country referred through its various outreach medical centres. The hospital runs an accredited microbiology laboratory.

Multidrug-resistant Enterobacteriaceae are often isolated in clinical specimens from both inpatients and outpatients at AKUHN. They are frequently implicated in infections involving the bloodstream, urinary tract, abdomen, skin and soft tissue. According to data on bloodstream infections (BSI) in the hospital from 2012 to 2014 only 30% of *Klebsiella pneumoniae* and 51% of *Escherichia coli* were susceptible to 3rd generation cephalosporins, while 59% and 41%, were susceptible to fluoroquinolones respectively (3).

Although some of the studies on antibiotic resistance in Gram negative rods conducted at this hospital have involved molecular techniques, few have addressed the issue of clonality among isolates within and between different locations in the hospital.

In the weeks leading to the month of August 2012 we noticed an increase in the number of MDR *K. pneumoniae* and *E. coli* and sought to determine the molecular mechanisms of resistance and clonality in these isolates at AKUHN.

Materials and Methods

In the month of August 2012 a total of 143 *E. coli* and 38 *K. pneumoniae* isolates were cultured from clinical specimens in the microbiology laboratory. Among these, 35 (24%) were MDR *E. coli*, and 19 (50%) MDR *K. pneumoniae* respectively. The patients who provided the samples ranged from less than a month to 90 years in age with a median of 38. There were more females (35) than males (19), which was attributed to a preponderance of UTIs in the females. Thirty of the isolates were from outpatients and 24 from inpatients. Multidrug-resistance was defined as non-susceptibility to one or more agents in three or more of the following antimicrobial agents: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, monobactams and penicillins.

Identification and susceptibility testing were performed using GNR and GN126 cards respectively on Vitek2 system (VITEK® 2 bioMérieux, France). The Advanced Expert System (AES) on Vitek2 configured the most likely phenotype for the majority of the isolates as extended spectrum beta-lactamase (ESBL). The Clinical

Laboratory and Standard Institutes (CLSI) guidelines for susceptibility breakpoints (version 2009) were used in this study.

DNA extraction

Fresh colonies were used for total bacterial DNA extraction using the ZR fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp. USA) according to the manufacturer's protocol, with a final elution volume of 50 μ L.

PCR

PCR for the common ESBL and carbapenemases genotypes *bla*_{CTX-M}; *bla*_{SHV}; *bla*_{TEM}; *bla*_{KPC}; *bla*_{SPM}; *bla*_{GES}; *bla*_{IMP}; *bla*_{NDM}; *bla*_{OXA}; and *bla*_{VIM} was performed using generic primers sourced from the University of Cape Town Oligonucleotide Synthesis Service (Table 1). Only isolates reported as non-susceptible to meropenem (MIC \geq 4 μ g/mL) were tested for the presence of carbapenemase genes. For each target gene, PCR amplification was carried out in a 50 μ L reaction volume containing 1.25 units Taq DNA polymerase, 1.5 mM MgCl₂, 0.2 μ M of each of the forward and reverse primers, 200 μ M of each dNTP, 12.5 μ L of PCR water, and 2.5 μ L of DNA template.

The PCR amplicons were subjected to gel electrophoresis on PS 500X (Hoefer Scientific instruments, USA) at 80 V for one hour, and then visualized using the Chemie Genius²Bio imaging system (Syngene, UK) after staining the gel with ethidium bromide.

Sequencing

The amplicons were sequenced using ABI3730xl (Life Technologies, USA) and the sequences assembled using the DNABaser software (Heracle BioSoft S.R.L, Romania). Sequences were compared to the published GenBank sequences using BLAST.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed according to the PulseNet protocol for *E. coli* (www.cdc.gov/pulsenet/pdf/ecoli-shigella-pfge-protocol-508.pdf) with modification-the restriction digestion time was extended to 3 hours at 37°C. Only 16 of the *E. coli* and 15 *K. pneumoniae* isolates were successfully typed.

The PFGE images were viewed in the Chemie Genius Bio imaging system (Syngene, UK) and analyzed using GelCompar software (Applied Maths, Belgium). Pulsotypes were derived by visualizing the bands and by computation based on the level of relatedness according to the DICE similarity coefficient and UPMGA. Optimization and tolerance limits were set at 1%.

Isolates displaying \geq 94% similarity (\approx 3 band difference) were assigned the same pulsotypes (26).

Results

The majority (70%) of isolates were obtained from outpatient urine samples (Table 2). Other locations included the wards and critical care units that provided a variety of specimens. *E. coli* was mainly isolated in UTIs in outpatients while *K. pneumoniae* was associated with a variety of infections in inpatients.

The isolates were resistant to the majority of the 18 antibiotics tested against with the exception of meropenem and amikacin (Figure 1).

PCR for the ESBL genes showed that overall *bla*_{CTXM} was present in 41 (74%) of the isolates, *bla*_{SHV} in 18 (33%) and *bla*_{TEM} in 13 (24%) (Table 2). There were notable differences between the two bacteria

species in the proportion of isolates harbouring SHV; *E. coli* none, *K. pneumoniae* 18 (94.7%), and TEM; *E. coli* 3 (8.6%) and *K. pneumoniae* 10 (52.6%). Sequence analysis showed that all CTX-M genes were *bla*_{CTX-M-15} and all TEM-related genes were *bla*_{TEM-1}. The predominant SHV-related genes were *bla*_{SHV-11}, 10 (55.6%) and *bla*_{SHV-12} 6 (33.3%). Others were *bla*_{SHV-2} and *bla*_{SHV-1} each present in a single isolate. *bla*_{SHV} was exclusively found in *K. pneumoniae* while *bla*_{CTXM} and *bla*_{TEM} were present in both of the microorganisms.

Nine (16.7%) of the isolates harboured all three ESBL genes and 8 of the isolates (14.8%) harboured two. Eight of the isolates (all *E. coli*) harboured none of the genes tested; all 8 of these isolates showed phenotypic resistance to 3rd generation cephalosporins.

Only two isolates harboured carbapenemases genes: one had *bla*_{NDM-1} and the other *bla*_{SPM}; both were *Klebsiella pneumoniae*.

Molecular typing was performed in 16 of *E. coli* and 15 of *K. pneumoniae* isolates. Only two *E. coli* isolates (E4 and E20) belonged to the same pulsotype, while all of the *K. pneumoniae* isolates belonged to separate pulsotypes (Figures 2 and 3).

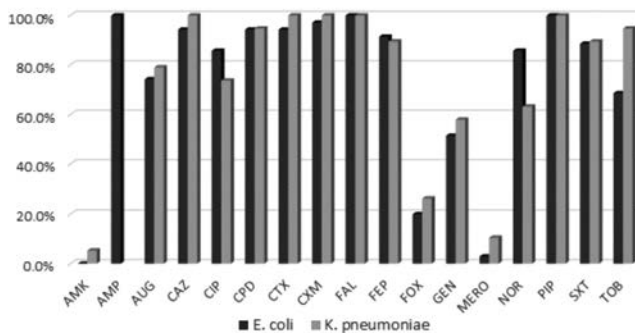


Figure 1. Proportion of isolates resistant to specific antibiotics (n=54). AMK amikacin; AMP ampicillin; AUG amoxicil/clavulanate; CAZ ceftazidime; CIP ciprofloxacin; CPD cefpodoxime; CTX cefotaxime; CXM cefuroxime; FAL cefalotin; FEP ceftazidime; FOX cefoxitin; GEN gentamicin; MERO meropenem; NOR norfloxacin; PIP piperacillin; SXT sulfamethoxazole/trimethoprim; TOB tobramycin.

Table 1. Primers used for detection of resistance genes.

Primer	Primer sequence (5'-3')	Annealing temperature	Amplicon size (bp)	Reference
SHV	F CGC CGG GTT ATT CTT ATT TGT CGC R TCT TTC CGA TGC CGC CGC CAG TCA	68°C	1016	(14)
TEM	F CTT CCT GTT TTT GCT CAC CCA R TAC GAT ACG GGA GGG CTT AC	58°C	717	(3)
CTX-M	F TTT GCG ATG TGC AGT ACC AGT AA R CGA TAT CGT TGG TGG TGC CAT A	51°C	544	(6)
KPC	F CGTCTAGTTCTGCTGTCTTG R CTTGTCATCCCTTGTAGGCG	55°C	798	(19)
SPM	F CCTACAATCTAACGGCGACC R TCGCCGTGTCCAGGTATAAC	55°C	650	(19)
GES	F CTATTACTGGCAGGGATCG R CCTCTCAATGGTGTGGGT	55°C	594	(19)
NDM	F GGTTTGCGGATCTGGTTTTC R CGGAATGGCTCATCACGATC	55°C	621	(19)
VIM	F GATGGTGTGGTTCGCATA R CGAATGCCGAGCACCAG	55°C	390	(19)
OXA-48	F GCGTGGTTAAGGATGAACAC R CATCAAGTTCAACCAACCG	55°C	438	(19)
IMP	F GGAATAGAGTGGCTTAAYTC R TCGGTTTAAAYAAACAACCACC	55°C	232	(19)

Table 2. Specimen type and resistance genes among the isolates.

	<i>E. coli</i> (n=35)	<i>K. pneumoniae</i> (n=19)
Specimen type: n (%)		
Urine	28 (80.0)	10 (52.6)
Respiratory	0 (0)	3 (15.8)
Pus / swabs	3 (8.6)	2 (10.5)
Blood Culture	3 (8.6)	2 (10.5)
Other	1 (2.9)	2 (10.5)
Beta lactamase: n (%)		
<i>bla</i> -CTX-M	26 (74.3)	15 (78.9)
<i>bla</i> -TEM	3 (8.6)	10 (52.6)
<i>bla</i> -SHV	0 (0)	18 (94.7)

Discussion

We analyzed the molecular characteristics of multidrug resistant *K. pneumoniae* and *E. coli* isolates collected during one month at AKUHN to determine the common ESBL and carbapenemase genes, as well as to describe the clonal relationship of the isolates. The predominant genotype was *bla*_{CTX-M-15} and was harboured by 41 (74%) isolates. This finding was similar to that of an earlier study carried out in the hospital involving community acquired infections

caused by the two organisms (10). The multidrug resistant isolates were present in all locations sampled in the hospital. Not surprisingly the majority of these isolates were implicated in urinary tract infections in the outpatient department. *bla*_{CTX-M-15} has a worldwide presence and is one of the most common variants detected in clinically important pathogens, and predominates in most of Europe, North America, the Middle East and India (8, 29). Data from Africa seem to suggest it is also prevalent in this continent. A study in the neighboring Tanzania on ESBL producing *K. pneumoniae* found that among 92 isolates, 68 (74%) had *bla*_{CTX-M-15} (13).

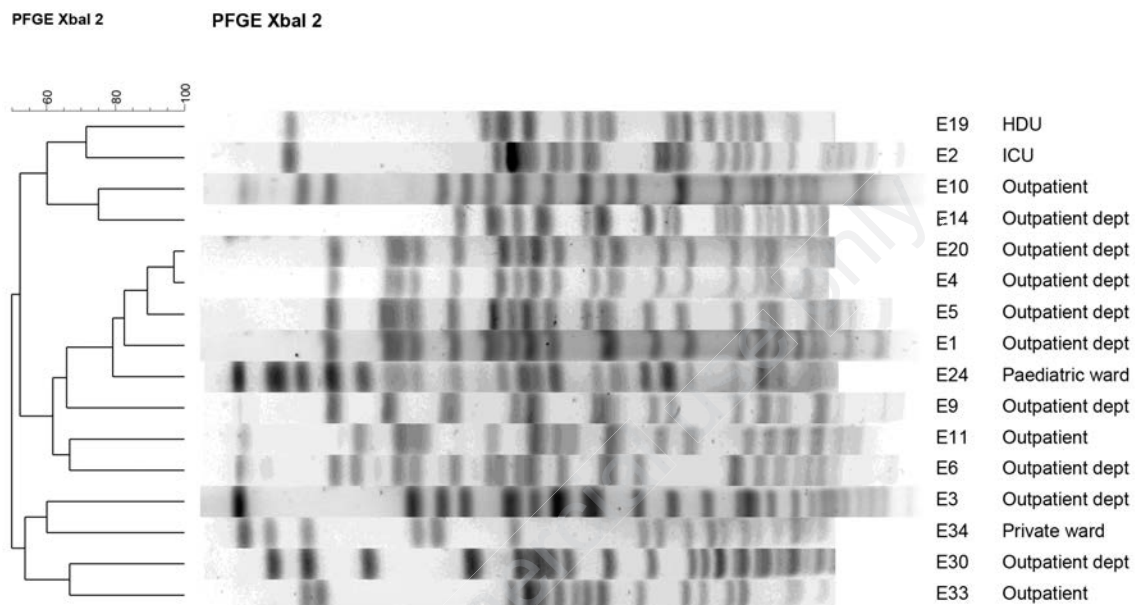


Figure 2. PFGE *E. coli*. HDU- high dependence unit; ICU- intensive care unit.

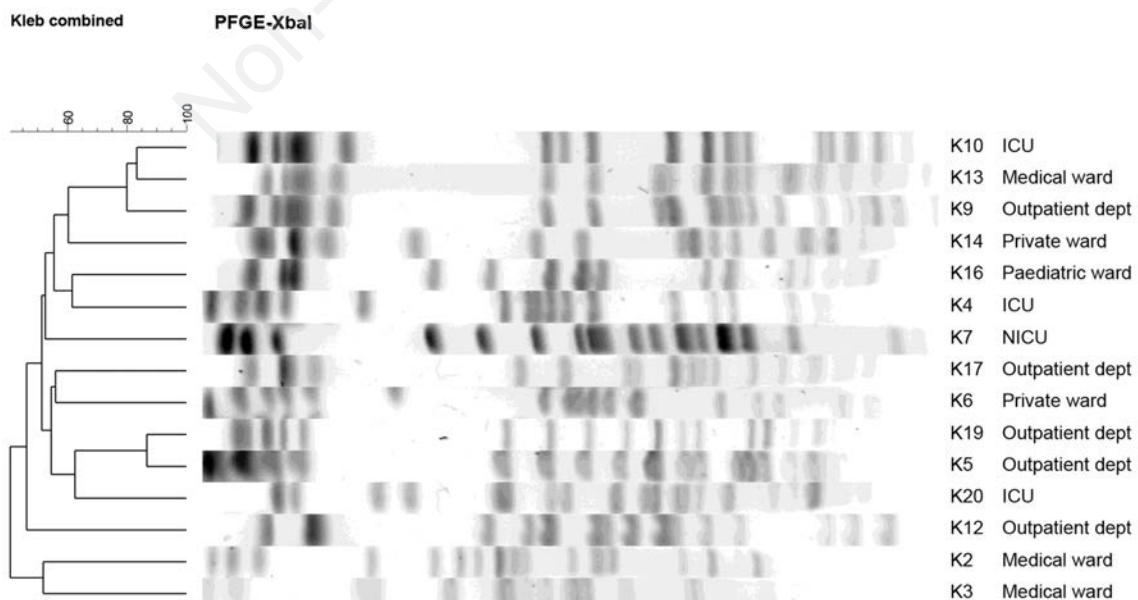


Figure 3. PFGE *K. pneumoniae*. HDU; high dependence unit; ICU- intensive care unit; NICU-neonatal intensive care unit.

Presence of the genotype *bla*_{CTX-M-15} has also been reported in other countries including Morocco, Tunisia, Algeria, Egypt, Nigeria and South Africa (16, 25). Apart from humans the environment as well as animals that are a food source serve as reservoirs for ESBLs and studies done on different continents have documented evidence, which in part explains the global presence and spread of ESBLs (1, 2, 12).

Eight of the isolates with phenotypic resistance to 3rd generation cephalosporins did not harbour any of the common ESBL genes tested. It is possible that other mechanisms of resistance, besides beta-lactamases, or other beta-lactamases whose genes were not tested for could be involved. One possible mechanism is the presence of AmpC beta-lactamases; however this seems unlikely considering that among the eight isolates only one was resistant to cefoxitin. Another plausible explanation is the presence of false positive ESBL phenotypes as determined by the automated system (Vitek2) since it is not 100% specific for ESBLs. Specificity varies between studies with some reporting above 97% (21, 24) while others report values lower than 90% (4, 7). In our study, confirmatory tests for ESBL production for the eight isolates were not performed.

Only 2 isolates, both *Klebsiella*, were found to harbour carbapenemase genes *i.e.* *bla*_{NDM-1} and *bla*_{SPM}.

*bla*_{NDM-1} which gained prominence in the last decade had been described in *K. pneumoniae* isolates dating back to 2007 in this hospital and later was also isolated in *Acinetobacter baumannii* (18, 20). However, carbapenemase producing *K. pneumoniae* and *E. coli* have remained relatively uncommon compared to ESBLs in Kenya. A systematic review that analysed carbapenemase-producing bacteria in Africa found diverse prevalence among hospital isolates ranging from 2.3% to 67.7%. The review concluded that antibiotic stewardship and surveillance systems, including molecular typing of resistant isolates, should be implemented to control the spread of carbapenemase-producing bacteria (11).

The SPM- type carbapenemase is a metallo-beta-lactamase (MBL) that is reported mostly in South America among *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*). There are limited data on the prevalence of the SPM-type carbapenemase in Africa. A study conducted in Uganda showed that 16% (4/25) of carbapenem-resistant *P. aeruginosa* harboured *bla*_{SPM} (5).

Although only a limited number of isolates were analysed by PFGE, results show the existence of different pulsotypes among the two species within the various hospital locations and makes clonal spread of the MDR isolates less likely. However, these studies need to be repeated to involve larger numbers of isolates collected over a longer time span to better understand the emergence and spread of the resistant isolates. Molecular investigations integrated with conventional hospital epidemiologic surveillance can be cost effective in reducing nosocomial infections (17). Clonality can be used to track the source and spread of an infection and a relapse from a re-infection. These information can lead to targeted infection control measures. However, the resources required to perform molecular studies are prohibitive in under-resourced countries- a situation that prevails in most of Africa.

The research reported here had limitations including low number of isolates typed and limited time of the study. However, we believe it provides a snapshot of what was happening in the hospital at the time, and can be used to assist future studies. Most importantly, it suggests that clonal dissemination of these resistant isolates is unlikely.

Conclusions

MDR *E. coli* and *K. pneumoniae* mostly harboured *bla*_{CTX-M-15}, and carbapenemase were uncommon. Among the isolates that underwent molecular typing no clonality was demonstrated suggesting they arose by means other than clonal spread, and were probably the stable circulating strains at the time of the study.

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