Phenotypic expression and prevalence of ESBL-producing Enterobacteriaceae in samples collected from patients in various wards of Mulago Hospital, Uganda

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Abstract

Background: Resistance to extended-spectrum cephalosporins among Enterobacteriaceae has been reported yet they serve as the last line treatment for severe infections in Uganda and other countries. This resistance often leads to nosocomial infection outbreaks and therapeutic failures from multidrug resistant bacteria. The main objective of this study was to determine the prevalence of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in clinical samples of patients in various wards of Mulago Hospital; Uganda’s main national referral and teaching hospital.

Methods: This cross-sectional study was conducted between January-April, 2014. Purposive consecutive sampling was used to collect pus swab, urine, blood and CSF samples from patients in the various wards. A total of 245 consecutive, non-repetitive, clinical samples were obtained and tested for phenotypic ESBL production using the Double Disc Synergy Test using cefotaxime, ceftazidime, cefotaxime-clavulanic acid and ceftazidime-clavulanic acid.

Results: Results show that 47 % of the 245 samples had Enterobacteriaceae isolates. Of these isolates 62 % were ESBL producers while 38 % were of non-ESBL phenotype. E. coli was the most isolated organism (53.9 %), followed by K. pneumoniae (28.7 %). Majority of Enterobacteriaceae organisms were isolated from urine samples, followed by pus samples and of these 64.9 % and 47.4 % were ESBL-producers respectively. Klebsiella pneumoniae had the highest percentage of ESBL producers (72.7 %). There was a higher percentage of isolates showing resistance to ceftazidime (73 %) compared to cefotaxime (57.5 %). All Enterobacter cloacae isolates showed resistance to ceftazidime. There were no statistically significant association between phenotype (ESBL/non-ESBL) and patients’ age or gender or Enterobacteriaceae spp.

Conclusions: This study reveals a high prevalence of ESBL producing organisms in Mulago Hospital and high levels of resistance to third generation cephalosporins. In addition to undertaking appropriate infection control measures, there is urgent need for formulation of an antibiotic policy in Uganda to prevent spread of these organisms. This also calls for continuous monitoring and reporting of the presence of such organisms in order to ensure rational and judicious use of antibiotics by clinicians.

Keywords: ESBL, Enterobacteriaceae, Cefotaxime, Ceftazidime, Clavulanic acid
Background
Production of β-lactamase enzymes that hydrolyze the β-lactam ring is a predominant resistance mechanism for many Gram-negative bacteria including Enterobacteriaceae such as *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter cloacae* and *Aeromonas spp.* [1]. Extended-Spectrum β-Lactamase (ESBL)-producing bacteria are capable of expressing these enzymes and this confers bacterial resistance to penicillins; first, second, and third-generation cephalosporins and aztreonam. ESBL-producing bacteria have been isolated in many parts of North America and Europe [2] and in Africa. In one South African hospital, 36% of *K. pneumoniae* isolates were ESBL-producers and outbreaks of infections due to *Klebsiella* strains resistant to third-generation cephalosporins have also been reported in Nigeria and Kenya [3, 4]. In Uganda there has been lack of published information about ESBL-producers among organisms isolated from patients.

ESBL producers have a wide clinical significance and potential impact in healthcare settings especially in low income countries such as Uganda. The selection pressure and overuse of new antibiotics in the treatment of patients leads to selection for new variants of β-lactamase producers. ESBL producers are associated with various infections in virtually all body organs leading to meningitis, pneumonia, urinary tract infections, septicemia and intra-abdominal infections [5, 6]. Other conditions include osteomyelitis, endophthalmitis, pyomyositis and wound infections [7].

The possible spread of ESBL-producing organisms in a clinical setting is real. ESBL-associated antibiotic resistance causes increased morbidity and mortality; and hampers the control of infectious diseases. This in turn leads to increase in durations of illness and hospital stay; increase in health-care costs and more economic burden to families. This study sought to determine the prevalence of ESBL-producing bacteria among isolates from samples in various wards of Mulago Hospital, the main national referral hospital in Uganda with a bed-capacity of 1,600.

Methods
Study design
This was a cross-sectional study conducted between January-April 2014 to determine the prevalence of ESBL-producing Enterobacteriaceae in clinical samples collected from in-patient and out-patient wards of Mulago Hospital. The samples collected included urine, swabs (oral, HVS, wound), blood and CSF.

ESBL n = 71 (61.7 %) Non-ESBL n = 44 (38.3 %) Total p value

| Sex   | ESBL  | Non-ESBL | Total | p value |
|-------|-------|----------|-------|---------|
| Female | 37 (52.1 %) | 30 (68.2 %) | 67 (58.3 %) | 0.089 |
| Male   | 34 (47.9 %) | 14 (31.8 %) | 48 (41.7 %) |       |
| Age    | 42 (SD 22) | 38 (SD 19) | Overall mean | 0.355 |

Table 2 Baseline characteristics of patients whose samples had Enterobacteriaceae isolates

The Chi-square and independent t-tests showed that sex and age were not significantly associated with ESBL phenotype.

Chi-square and independent t-test

Table 3 Enterobacteriaceae species isolated from patient samples

| Isolates                  | Frequency | Percent |
|---------------------------|-----------|---------|
| *E. coli*                 | 62        | 53.9    |
| *Klebsiella pneumoniae*   | 33        | 28.7    |
| *Proteus mirabilis*       | 16        | 13.9    |
| *Enterobacter cloacae*    | 4         | 3.5     |
| Total                     | 115       | 100.0   |

Table 1 Criteria for determining the potency of the test antibiotics

| ESBL Sample | E. coli ATCC 25922 | *K. pneumoniae* ATCC 700603 |
|-------------|--------------------|-----------------------------|
| Cefazidime  | (25-32 mm)        | (22-29 mm)                  |
| Cefotaxime  | (29-35 mm)        | (18-22 mm)                  |

The antibiotics had to show inhibition zone diameters in the above ranges in order to be used in the study.

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| Total                     | 115       | 100.0   |

*E. coli* was the predominant isolate.
labelled and taken to the Microbiology Laboratory for Enterobacteriaceae culture and isolation. The originating ward, patient’s gender and age were recorded. The samples were inoculated by streaking on Blood agar (Oxoid, UK), MacConkey agar (Oxoid, UK) and CLED agar (Oxoid, UK) plates. The plates were incubated aerobically at 37 °C for 18–24 h to allow development of bacterial colonies. Preliminary identification of the isolates was done using phenotypic colonial characteristics. Confirmatory identification of the suspect colonies was carried out by conventional biochemical tests as described by Cheesbrough [10]. These were: indole, Methyl red, Voges-Proskauer, citrate utilization and urease production tests as well as triple sugar iron and oxidase tests.

Detection of ESBL-producing Enterobacteriaceae
ESBL detection was based on the Double Disc Synergy Test and interpretation of the results done using the CLSI M100-S20 (2010) [11]. Briefly; 3–5 colonies of each isolate were picked from the growth plates with sterile wire loop, and suspended in 1 ml of physiological saline. The resultant bacterial suspension was matched to the 0.5 McFarland turbidity standard so as to approximate the seeding density of the respective organisms. 100 µl of the bacterial suspension/broth culture were then surface-spread on Muller Hinton Agar (Oxoid, UK) plates using a sterile spreader. Antibiotic discs containing ceftazidime (CTC), cefotaxime (CTZ), ceftazidime-clavulanic acid (CFC) and cefotaxime-clavulanic acid (CTX) were placed on the plates which were incubated overnight at 37 °C. The zones of clearance (mm) for the respective antibiotics were measured for each isolate using a divider and ruler. Organisms were considered to be ESBL-producers if the difference in zone of clearance between ceftazidime and ceftazidime-clavulanic acid or cefotaxime and cefotaxime-clavulanic acid was ≥5 mm. The prevalence of ESBL-producing bacteria was determined using the formula:

\[
\text{Prevalence (P)} = \frac{\text{Number of ESBL producing organisms}}{245} \times 100
\]

Quality control
Standard organisms (E. coli ATCC 25922 and K. pneumoniae ATCC 700603) were used to test for the potency of the antimicrobial discs (Table 1).

Data analysis
Clinical and socio-demographic data were entered into Epi Info™ v7 and exported to SPSSv21. Pearson Chi-square test was used to assess for any differences between the two ESBL phenotype categories with respect to clinical and demographic parameters. The means of the continuous variables, age and zones of clearance were...
compared using the Independent t-test. Crude logistic regression analysis was used to explore clinical and laboratory features of the ESBL phenotype for comparison with non-ESBL Enterobacteriaceae phenotypes. The differences were considered significant at p < 0.05.

Ethical considerations

The study protocol was approved by the Ethics Review Committee of the School of Biomedical Sciences of Makerere University Medical School. Permission was sought from the hospital and laboratory authorities. The ethical principles of scientific research as well as related national laws and regulations were strictly adhered to.

Results

The mean age of the participants was 40 years as shown in Table 2. Results indicated that 115 of the 245 samples (47 %) had Enterobacteriaceae isolates. Of these isolates, 58.3 % were from female patients while 41.7 % were from males (Table 2). Statistical analysis of patient data using Chi-square and independent t-test indicated that gender and age were not significantly associated with ESBL phenotype.

E. coli was the most isolated organism (53.9 %, n = 62), followed by K. pneumoniae (28.7 %) as shown in Table 3. Most samples with Enterobacteriaceae isolates were from Obstetrics and Gynaecology wards i.e. 5A (10.4 %, n = 12) and 5C (11.3 %, n = 13) (Fig. 1).

Results further showed that 62 % of Enterobacteraeae isolates were of the ESBL phenotype while 38 % were of non-ESBL phenotype (Table 2). Most of the Enterobacteriaceae were isolated from the urine samples followed by pus samples as shown in Table 4 and Fig. 2. However, just 64.9 % and 47.4 % of urine and pus isolates respectively were ESBL-producers. On the other hand,
Enterobacter cloacae
E. coli
26 mm)
E. coli
Susceptibility pattern of Enterobacteriaceae to ceftazidime and cefotaxime. [15] in Tanzania (45.2 %). This wide vari-
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(53.8 %). Similar find-
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clavulanic acid (25.1 ± 6.6 mm; 22.3 ± 8.3 mm respectively) as shown in Table 5. The zones of clearance of ceftazidime and cefotaxime were significantly associated with ESBL phenotype (p = 0.000; 0.000 respectively) while those of ceftazidime-clavulanic acid and cefotaxime-clavulanic acid were not (p = 0.198, 0.051 respectively).

There was a higher percentage of isolates showing resistance to ceftazidime (73 %) compared to cefotaxime (57.5 %) as shown in Table 6. All Enterobacter cloacae isolates were resistant to both ceftazidime and ceftazi-
dime. Table 4 shows that Klebsiella pneumoniae had the highest percentage of ESBL producers (72.7 %). There were no statistically significant association between phenotype (ESBL/non-ESBL) and patients’ age or gender (Table 4).

Similarly, there were no significant association between phenotype (ESBL/non-ESBL) and species of Enterobacteri-
aceae (E. coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis) or sample type (Table 4).

Discussion
The 3rd and 4th generation cephalosporins are often reserved for severe infections [12] but resistance to these drugs has been strikingly rapid worldwide [4]. Consequently, therapeutic options for the infections caused by the ESBL producers are becoming increasingly limited and; if available, expensive for low and middle income countries. The study revealed a slight female preponder-
ance for ESBL-producing Enterobacteriaceae among the patients though gender was not statistically significant (p = 0.089) as a factor. Kiratisin et al., [13] also revealed a female preponderance.

Urine samples constituted the greatest number of clinical samples in this study. According to Wilson and Gaido [14], urinary tract infections constitute the commonest bacterial infections and urine samples account for a sign-
ificant percentage of samples in clinical microbiology lab-
oratories worldwide. Most Enterobacteriaceae isolates were E. coli. Wilson and Gaido [14] indicates that E. coli is the most frequent cause of urinary tract infections and this could probably explain the high prevalence of E. coli iso-
lates in our study. Similarly, studies in Tanzania [15] indi-
 cate that E. coli and Klebsiella pneumoniae are the most prevalent Enterobacteriaceae species in clinical samples. Furthermore, Maina et al., [16] in a study in Kenya re-
ported higher prevalence for E. coli (53.8 %). Similar find-
ings were reported in Bahrain [17].

In our study, the highest numbers of ESBL-producing isolates were from gynaecological and surgical wards. Many studies associate ESBL-producing Enterobacteri-
aceae with surgical wards. Studies by Seni et al., [9] show that most isolates from surgical wards are ESBL-producers. Prolonged hospital stay; inappropriate therapy; use of ind-
dwelling catheters, endotracheal/nasogastric tubes and se-
vere illnesses are all possible drivers of their dissemination. Significantly, there is also movement of health workers between wards in the hospital and can migrate ESBL-
producers from ward to ward leading to dissemination throughout the hospital.

The prevalence of ESBL producers among Enterobacteriaceae (62 %) was quite high compared to that reported by Moyo et al., [15] in Tanzania (45.2 %). This wide vari-
ation in prevalence is probably due to differences in types of samples analysed and the extent of antibiotic use in the various wards. The present study reveals that Klebsiella pneumoniae and E. coli are major ESBL producers. Moyo et al., [15] showed 51.5 % and 39.1 % ESBL positivity among Klebsiella spp and E. coli respectively. On the other hand,

Table 5 Mean zone of clearance (± SD) of Enterobacteriaceae segregated by ESBL phenotype

| Antibiotic                  | ESBL           | Non-ESBL        | P value |
|-----------------------------|----------------|-----------------|---------|
| Ceftazidime                 | 10.1 ± 6.2     | 18.1 ± 7        | 0.000   |
| Cefotaxime                  | 13.4 ± 8.3     | 21.6 ± 8.3      | 0.000   |
| Ceftazidime-Clavulanic acid| 22.3 ± 6       | 20.7 ± 6.8      | 0.198   |
| Cefotaxime-Clavulanic acid  | 25.1 ± 6.6     | 22.3 ± 8.3      | 0.051   |

The zones of clearance of ceftazidime and cefotaxime were statistically significantly associated with ESBL phenotype (Independent samples t-test).

albeit their small number, all isolates from CSF, wound and urethral swabs were ESBL producers (Fig. 2).

The mean zones of clearance for ESBL and non-ESBL phenotypes were lowest for ceftazidime (10.1 ± 6.2 mm; 18.1 ± 7 mm respectively) and highest for cefotaxime-clavulanic acid (25.1 ± 6.6 mm; 22.3 ± 8.3 mm respectively) as shown in Table 5. The zones of clearance of ceftazidime and cefotaxime were significantly associated with ESBL phenotype (p = 0.000; 0.000 respectively) while those of ceftazidime-clavulanic acid and cefotaxime-clavulanic acid were not (p = 0.198, 0.051 respectively).

Table 6 Susceptibility pattern of Enterobacteriaceae to ceftazidime and cefotaxime

| Antibiotic | Ceftazidime (%) | Cefotaxime (%) |
|------------|-----------------|----------------|
|            | Resistant       | Intermediate   | Susceptible   | Resistant       | Intermediate   | Susceptible   |
| E. coli    | 41 (66.1)       | 7 (11.3)       | 14 (22.6)     | 32 (51.6)       | 9 (14.5)       | 21 (33.9)     |
| E. cloacae | 4 (100)         | 0 (0)          | 0 (0)         | 3 (75)          | 0 (0)          | 1 (25)        |
| K. pneumoniae | 27 (81.8) | 1 (3)          | 5 (15.2)      | 21 (63.6)       | 3 (9.1)        | 9 (27.3)      |
| Proteus mirabilis | 12 (75) | 3 (18.8) | 1 (6.3) | 10 (62.5) | 1 (6.3) | 5 (31.2) |
| %          | 73.0            | 9.6            | 17.4          | 57.5            | 11.3           | 31.3          |

There was a higher percentage of Enterobacteriaceae isolates showing resistance to ceftazidime than to cefotaxime. The interpretative criteria used was based on CLSI M100-S20 (2010) [11] where for ceftazidime (Resistant ≤17 mm; Intermediate 18-20 mm; Susceptible ≥21 mm) and for cefotaxime (Resistant ≤22 mm; Intermediate 23-25 mm; Susceptible ≥26 mm).
Seni et al., [9] reported that 79.2 % and 92.3 % of E. coli and K. pneumoniae isolates are ESBL producers; further evidence that these two organisms account for most ESBL producers in the region. Our study showed a higher resistance to cefazidime than to cefotaxime. On the other hand, Maina et al., [16] reported 21.2 % resistance to cefazidime and 65.4 % resistance to cefotaxime. The differences seen in this study could be due to regional differences and the type of samples collected.

Conclusions
This study has demonstrated high prevalence of ESBL-producing Enterobacteriaceae in Mulago Hospital. The spread of these organisms reduces the antibiotic alternatives for the treatment of infections by these pathogens to mainly carbapenems; which are often reserved for life-threatening infections. The study underscores the need for routine detection and reporting of ESBL-producers in Ugandan medical facilities so that measures are taken to avoid their uncontrolled spread and possible therapeutic failures. Clinicians need to be rational and judicious in use of antibiotics. An antibiotic use policy is also imperative to limit the dissemination of these organisms.

Abbreviations
ATCC: American type culture collection; Cl: Confidence interval; CLED: Cystine Lactose-Electrolyte-Deficient (agar); CSF: Cerebro-spinal fluid; ESBL: Extended spectrum beta-lactamase; HVS: High vaginal swab.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JNK, MLN and RK conceptualized the project, performed most of the lab experiments and wrote the manuscript. MLN contributed specific knowledge in conduction of the microbiological assays. CA assisted in statistically analysing the data. JGN assisted in finalizing the manuscript. All authors read and approved the final manuscript.

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