





RESEARCH ARTICLE

Phylogenetic groups and antimicrobial susceptibility patterns of uropathogenic *Escherichia coli* clinical isolates from patients at Mulago National Referral Hospital, Kampala, Uganda [version 1; peer review: awaiting peer review]

Paul Katongole ¹, Daniel Bulwadda Kisawuzi¹, Henry Kyobe Bbosa ²,
David Patrick Kateete³, Christine Florence Najjuka¹

¹Department of Medical Microbiology, Makerere University, College of Health Sciences, KAMPALA, Kampala, 7072, Uganda

²African Risk Capacity, Pretoria, South Africa

³Department of Immunology and Molecular Biology, Makerere University, College of Health Sciences, KAMPALA, Kampala, 7072, Uganda

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Abstract

Introduction: Uropathogenic *Escherichia coli* (UPEC) remains the most common cause of urinary tract infections (UTIs). They account for over 80-90% of all community-acquired and 30-50% of all hospital-acquired UTIs. *E. coli* strains have been found to belong to evolutionary origins known as phylogenetic groups. In 2013, Clermont classified *E. coli* strains into eight phylogenetic groups using the quadruplex PCR method. The aim of this study was to identify the phylogenetic groups of UPEC strains in Uganda using Clermont's quadruplex PCR method and to assess their antibiotic susceptibility patterns in Uganda.

Methods: In this cross-sectional study, 140 stored uropathogenic *E. coli* isolates from the Clinical Microbiology Laboratory, Department of Medical Microbiology, College of Health Sciences Makerere University were subjected to phylogenetic typing by a quadruplex PCR method. Antimicrobial susceptibility testing was performed by disk diffusion method according to Clinical & Laboratory Standards Institute (CLSI) guidelines. Phenotypic detection of extended-spectrum beta-lactamase, AmpC and carbapenemases was done according to CLSI guidelines and Laboratory SOPs.

Results: Phylogenetic group B2 (40%) was the most predominant, followed by A (6.23%), clade I and II (5%), D and E (each 2.14%), B1 (1.43%) and F and C (each 0.71%). The most common resistant antibiotic was trimethoprim-sulphamethoxazole (90.71%) and the least was imipenem (1.43%). In total, 73.57% of isolates were multi-drug resistant (MDR). Antibiotic resistance was mainly detected in phylogenetic group B2 (54%).

Conclusions: Our findings showed the high prevalence of MDR *E. coli* isolates, with the dominance of phylogenetic group B2. About 9% of *E. coli* isolates belonged to the newly described phylogroups C, E, F, and clade I and II.

Open Peer Review

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Uropathogenic E.coli, Phylogenetic groups, Clermont's, Quadruplex PCR, Antimicrobial resistance.

Corresponding author: Paul Katongole (paul.katongole@gmail.com)

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Introduction

Urinary tract infections (UTIs) remain a major cause of morbidity, with over 1.5 million annual cases reported worldwide (Lee & Neild, 2007)(Neild, 2003). *Escherichia coli* is the major cause of UTIs accounting for over 80–90% and 30–50% of all community-acquired and hospital-acquired UTIs respectively(Foxman, 2010). Phylogenetic (evolutionary) groups of *E. coli* strains have been shown to differentiate between pathogenic and commensal strains depending on their fitness landscapes and virulence characteristics. Although multi-locus sequence typing (MLST) and ribotyping are the gold standard methods for phylogenetic typing of *E. coli* strains, these methods are expensive, time-consuming and require the collection of typed strains (Clermont *et al.*, 2000).

In 2000, Clermont *et al.* (Clermont *et al.*, 2000). developed a triplex PCR assay that classified *E. coli* strains into four different phylogenetic groups, A, B1, B2 and D based on the presence or absence of two genes, namely *chuA*, *yjaA*, and one DNA fragment *TspE4.C2*. Since 2000, growing knowledge of MLST for *E. coli* from different habitats has made it possible to validate the triplex PCR method (Gordon *et al.*, 2008). The validation studies found that only 80–85% of all *E. coli* phylogenetic groups were assigned correctly(Gordon *et al.*, 2008)(Clermont *et al.*, 2013).

In 2013 Clermont *et al.* (Clermont *et al.*, 2013) added an additional gene target, *arpA*, to the three candidate markers (*chuA*, *yjaA* and *TspE4.C2*) and developed a quadruplex PCR assay to classify *E. coli* isolates into eight phylogroups: A, B1, B2, C, D, E, F, and clade III. The use of this quadruplex PCR phylotyping method has been found to correctly assign 95% of all *E. coli* strains. Phylogenetic analysis has demonstrated a relationship between different *E. coli* phylogenetic groups, antimicrobial resistance and other virulence characteristics (Iranpour *et al.*, 2015). Different studies have shown that the most virulent and antimicrobial-resistant extra-intestinal *E. coli* strains belong mainly to group B2 and, to a lesser extent, to group D (Iranpour *et al.*, 2015)(Bashir *et al.*, 2011)(Liu *et al.*, 2014). In contrast, most of the commensal strains are associated with group A or group B1 (Clermont *et al.*, 2000). In this study, we aimed to determine the prevalence of the different phylogenetic groups of Uropathogenic *E. coli* strains using the new Clermont quadruplex PCR phylotyping method and their antimicrobial susceptibility patterns.

Methods

Study design and setting

This was a cross-sectional laboratory-based study carried out in the Clinical Microbiology and Molecular Biology Laboratories in the Department of Medical Microbiology, College of Health Sciences, Makerere University (Kampala, Uganda).

Bacterial strains

A total of 140 Uropathogenic *E. coli* strains that belonged to the bacterial collection of the Department of Medical Microbiology, College of health sciences Makerere University were studied. These strains had been isolated and stored at -80°C during a 12-month period (between January and December

2016). These isolates had been recovered from samples with a bacterial count of 10⁵ CFU/ml of midstream urine samples of patients with a suspected UTI. The samples were selected by consecutive sampling and all samples that were poorly labeled and lacked traceable laboratory request form were excluded from the study. All isolates were plated on MacConkey agar and incubated at 37°C aerobically for 18–24 hours to obtain pure growth.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done using the Kirby Bauer disk-diffusion method. Briefly, between one and five colonies of the isolate from the MacConkey agar were emulsified in saline and adjusted for turbidity to obtain a 0.5 McFarland standard. A sterile cotton swab on a stick was dipped in the colony-saline mixture, excess saline was then squeezed out by pressing the swab against the test tube, and the cotton swab gently applied onto the surface of the Mueller-Hinton-2- agar to obtain a uniform lawn. Up to six antibiotic impregnated discs were gently placed on the agar surface, at a minimum distant of 25 mm from each other, and the plates incubated at 37°C aerobically for 18–24 hours. The zones of inhibition diameters around each disc were measured using a ruler and compared against the zone diameter interpretative standards according to CLSI (2014). A panel of 12 antibiotics were used: ampicillin (30 µg), cefuroxime (30 µg), amoxicillin/clavulanic acid (20/10 µg), gentamycin (10 µg), trimethoprim/sulphamethoxazole (1.25/23.5 µg), chloramphenicol (5 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), ceftazidime (30 µg), imipenem (10 µg), nalidixic acid (30 µg) and nitrofurantoin (300 µg). *E. coli* ATCC 25922 was used as a control strain.

ESBL detection

All isolates that showed an area of inhibition of diameter <22 mm for ceftazidime and <25 mm for ceftriaxone, which were selected for phenotypic detection of The test isolate was mixed in saline to obtain a suspension of 0.5 McFarland standard. The suspension was later swabbed to make a uniform lawn on Mueller-Hinton agar (MHA) plate. An amoxicillin-clavulanate (Augmentin) (20 µg/10 µg) disk was placed in the center of the plate with a 30-µg disk of a third-generation cephalosporin (ceftazidime and cefotaxime) at a distance of 20 mm from center to center on a Mueller Hinton agar (MHA) plate on opposite sides. The plate was later incubated aerobically at 37°C for 18–24 hours. All isolates that showed a clear extension of the edge of inhibition zone of the third-generation cephalosporin toward the augmentin disk were interpreted as positive for ESBL production.

Carbapenemase detection

Detection of carbapenemases was determined by a positive Modified Hodge test (MHT) as described by CLSI (2014); In brief, *E. coli* ATCC 25922 was streaked for confluent growth on Mueller-Hinton II agar plates. A disk saturated with 10 µg of imipenem was placed in the center of the plate, and each sample was then subsequently streaked from the disk to the edge of the plate. The presence of a distorted inhibition zone after overnight incubation was interpreted as a positive result. *Klebsiella pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC

BAA- 1706 were used as positive and negative controls, respectively. Phenotypic detection of Metallo-beta-lactamases (MBL), *K. pneumoniae* producing carbapenems (KPC) and AmpC was carried out as described by Andrea Bartolini *et al* and Tsakris *et al* (Bartolini *et al.*, 2014)(Tsakris *et al.*, 2008).

DNA extraction

DNA for amplification was extracted from whole cells by the boiling lysis method, as explained briefly below. A full loop of pure colonies from fresh pure cultures was suspended in 1 ml of sterile distilled water. The cells were lysed by heating at 95°C for 10 minutes. The cells were then vortexed for 5 seconds. Centrifugation was later done at 13,000 rpm for 5 minutes at room temperature and the sample kept at -20°C to harvest the supernatant containing the DNA. The supernatant was subsequently used for PCR as template DNA. The integrity of extracted DNA was evaluated by electrophoresis on a 1% agarose gel. The purity of DNA was also determined by the ratio A260/A280 using a spectrophotometer.

New Clermont's quadruplex PCR Phylotyping

The distribution of phylogenetic groups amongst *E. coli* isolates was determined by the New Clermont Quadruplex PCR phylotyping method of 2013 (Clermont *et al.*, 2013). Briefly, a single reaction mixture contained 2 µL of 10x buffer (supplied with *Taq* polymerase), 2 µL of DNA (approximately 100 ng), 20 pmol of each appropriate primer (except for *AceK.f* (40 pmol), *ArpA1.r* (40 pmol), *trpBA.f* (12 pmol), and *trpBA.r* (12 pmol)) (Shanghai Genaray Biotech Co., Ltd.), 2mM of each dNTP, and 2U of *Taq* DNA polymerase (Fermentas, Lithuania) in a total volume of 20 µL. Primer sequences for the new Clermont's quadruplex PCR phylogroup assignment method are as shown in Table 1. PCR amplifications were carried out on a thermal cycler Master-cycler gradient (Eppendorf, USA) under

the following conditions: initial denaturation at 94°C for 4 min and 30 cycles for each denaturation at 94°C for 5 sec, annealing at 57°C for 20 sec (group E) or 59°C for 20 sec (quadruplex and group C), amplification at 72°C for 1 min, and final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis with Qiagel machine and a 2% agarose gel, stained with DNA safe stain (CinnaGen, Tehran, Iran) and visualized using GelDoc 2000 transilluminator (Bio-Rad Laboratories, Milan, Italy). A molecular weight standard (100-bp ladder, Fermentas, Lithuania) was used.

Statistical analysis

The data was entered in an excel spreadsheet, cleaned and double-checked for missing variables duplicate entries and values out of range. The data was then exported to STATA version 14 for statistical analysis. Means and proportions were obtained. Chi-square test or the Fisher exact test was applied to compare categorical variables. *P* values < 0.05 were considered to be statistically significant.

Ethical considerations

Ethical approval (SBS-HDREC- 487) and a waiver of consent were sought to use stored clinical isolates from the higher degrees Research and Ethics Committee of the School of Biomedical Sciences, College of Health Sciences Makerere University and the Uganda National Council of Science and Technology.

Results

Demographics from the isolate data

Of the 140 *E. coli* isolates, 102 (72.9%) were females and 38 (27.1%) were males. The age of the patients was ranging from 2 to 91 years. The mean age was 36.27 with a standard deviation of 18.98.

Table 1. Primer sequences that were used in the Clermont's quadruplex phylotyping method.

PCR Reaction	Primer ID	Target	Primer Sequence	Product length (bp)
Quadruplex	<i>chuA.1b</i>	<i>ChuA</i>	5-ATGGTACCGGACGAACCAAC-3	288
	<i>chuA.2</i>		5-TGCCGCCAGTACCAAAGACA-3	
	<i>yjaA.1b</i>	<i>yjaA</i>	5-CAAACGTGAAGTGTCAGGAG-3	211
	<i>yjaA.2b</i>		5-AATGCGTTCCTCAACCTGTG-3	
	<i>TspE4C2.1b</i>	<i>TspE4.C2</i>	5-CACTATTTCGTAAGGTCATCC-3	152
	<i>TspE4C2.2b</i>		5-AGTTTATCGCTGCGGGTCGC-3	
	<i>AceK.f</i>	<i>arpA</i>	5-AACGCTATTCGCCAGCTTGC-3	400
	<i>ArpA.r</i>		5-TCTCCCATACCGTACGCTA-3	
Group E	<i>ArpAgpE.f</i>	<i>arpA</i>	5-GATTCCATCTTGTCAAAATATGCC-3	301
	<i>ArpAGPE.r</i>		5-GAAAAGAAAAAGAATCCCAAGAG-3	
Group C	<i>trpAgpC.1</i>	<i>trpA</i>	5-AGTTTTATGCCAGTGCAGAG-3	219
	<i>TRpAgpC.2</i>		5-TCTGCGCCGGTCACGCC-3	
Internal control	<i>trpBA.f</i>	<i>trpA</i>	5-CGGCGATAAAGACATCTTAC-3	489
	<i>trpBA.r</i>		5-GCAACGCGGCCTGGCGGAAG-3	

Culture and susceptibility results

Resistance was highest to trimethoprim-sulphamethoxazole 127/140 (90.71%) followed by ampicillin 122/140 (87.14%) while resistance to nitrofurantoin 16/140 (11.43%) and imipenem 2/140 (1.43%) was minimal. Resistance to other antibiotics were as follows; cefuroxime 72/140 (51.43%), amoxicillin-clavulanic acid 46/140 (32.86%), gentamycin 52/140 (37.14%), chloramphenicol 35/140 (25%), ciprofloxacin 80/140 (57.14%), ceftriaxone 69/140 (49.29%), ceftazidime 50/140 (35.71%) and nalidixic acid 94/140 (67.14%). **Figure 1** contains a bar chart depicting percentage resistance to different antibiotics. Data

from disc-diffusion assays are available as *Underlying data* (Katongole, 2019).

Multi-drug resistant (MDR) organisms

In the study, 103/140 isolates (73.57%) were found to be MDR. In addition, 4/140 isolates (2.85%) were resistant to all antibiotics tested.

ESBL, AmpC, and carbapenem detection

In this study, 61/140 (43.57%) were positive for ESBL production. Out of all the ESBL positive isolates, (57/61) 93.44% were

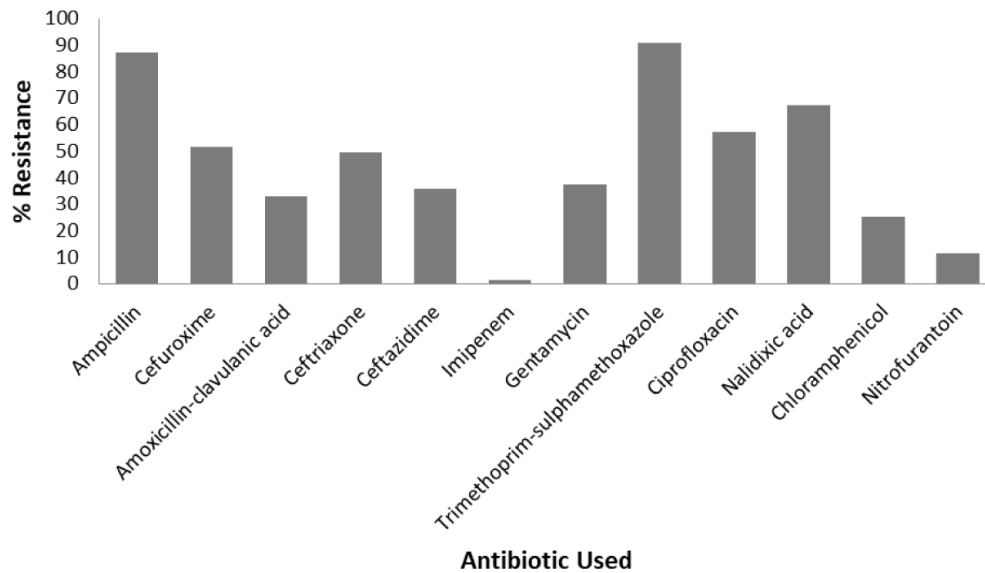


Figure 1. Percentage resistance of uropathogenic *E. coli* clinical isolates to different antibiotics (N=140).

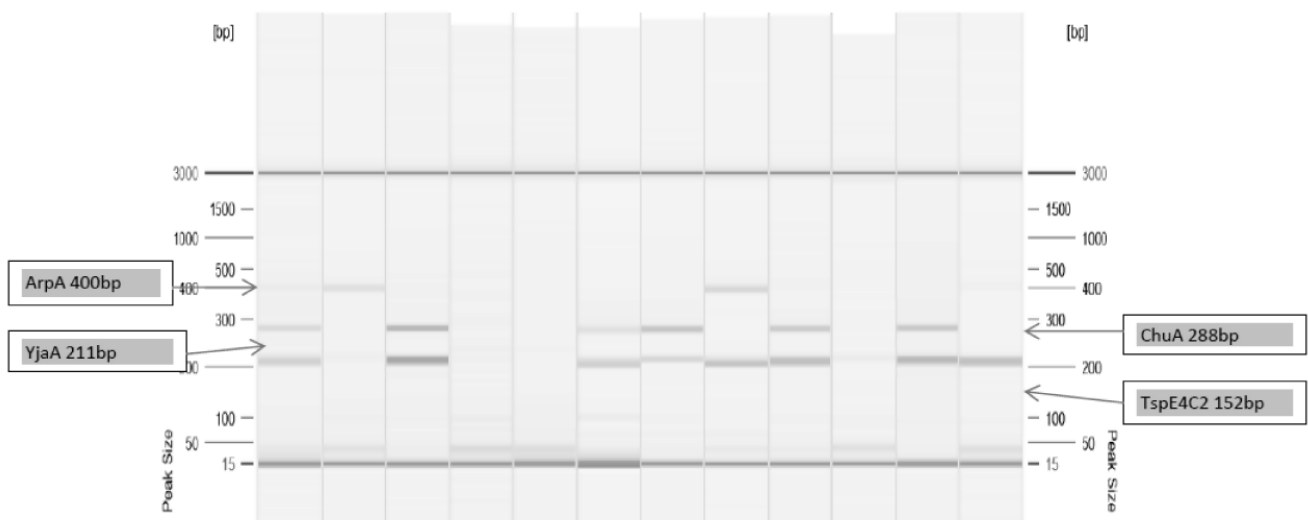


Figure 2. Quaxel gel Picture of Clermont's Quadruplex PCR of some Uropathogenic *E. coli* isolates. Shown are bands for different genes for phylogenetic typing

MDR. All isolates were negative for carbapenem production. Only 2/140 (1.43%) were AmpC producers.

Phylogenetic grouping

Phylogenetic group B2, in 56/140 of samples (40%), was the most prevalent, followed by group A in 9/140 samples (6.23%), Clade I/II in 7/140 samples (5%). The least prevalent were groups F (1/140 samples, 0.71%) and C (1/140 samples, 0.71%) (Table 2). Of the total isolates, 41.43% were unknown. Phylogenetic groupings for each patient are available as *Underlying data* (Katongole, 2019).

Table 2. Prevalence of the phylogenetic groups of Uropathogenic *E. coli* isolates.

Phylogenetic group	Prevalence, n (total 140)	Prevalence, %
A	9	6.23
B1	2	1.43
B2	56	40
C	1	0.71
D	3	2.14
E	3	2.14
F	1	0.71
Unknown	58	41.43
Clade I or II	7	5

Phylogenetic groups and Antimicrobial resistance

Phylogenetic group B2 was the most frequently resistant (38.57%). Phylogenetic groups B1 and F were the least resistant with a recorded highest resistance rate of 0.71% (Table 3).

Discussion

The clinical management of UTIs is becoming a major burden due to the emergence of MDR uropathogens (Shabbir *et al.*, 2018)(Flores-Mireles *et al.*, 2015). Currently, third-generation cephalosporins are the most commonly used drugs in the management of complicated and uncomplicated UTIs (Stiller *et al.*, 2017)(Bonkat *et al.*, 2017). This study aimed to identify the phylogenetic groups of UPEC clinical isolates based on the Clermont quadruplex PCR method and to assess the relationship between these phylogroups and antibiotic susceptibility patterns in Uganda. In this study majority of the *E. coli* strains belonged to B2 (40%). This was similar to other studies worldwide (Iranpour *et al.*, 2015)(Basu *et al.*, 2013)(Moreno *et al.*, 2008)(Takahashi *et al.*, 2006). Other studies have, however, the most predominant phylogenetic group was group A (Ejrnæs *et al.*, 2011)(Zhao *et al.*, 2015). Phylogenetic group B2 has been associated with MDR-UPEC strains and increased expression of virulence factors (Ochoa *et al.*, 2016)(Molina-López *et al.*, 2011)(Nüesch-Inderbinen *et al.*, 2017). Persistent and recurrent UTIs have also been associated with phylogenetic group B2 and this has been implicated in the pathogenesis of pyelonephritis(Ejrnæs *et al.*, 2011)(Kudinha *et al.*, 2013)(Luo *et al.*, 2012). A similar study by Ramos *et al.* of pregnant mothers at Mulago National Referral Hospital found B1 to be the most predominant phylogenetic group (Ramos *et al.*, 2012). The difference could be explained by the different study

Table 3. Prevalence of resistance among different phylogenetic groups of *E. coli* isolate.

Antibiotics	Phylogenetic groups								
	A N (%)	B1 N (%)	B2 N (%)	C N (%)	D N (%)	E N (%)	F N (%)	Clade I/II N (%)	Unknown N (%)
Amoxicillin	7 (5)	1 (0.71)	50 (35.71)	1 (0.71)	2 (1.43)	3 (2.14)	1 (0.71)	7 (5)	50 (35.71)
Cefuroxime	3 (2.14)	0 (0.0)	33 (23.57)	1 (0.71)	0 (0.0)	1 (0.71)	0 (0.0)	2 (1.43)	32 (22.86)
Amoxicillin-clavulanic acid	2 (1.43)	0 (0.0)	19 (13.57)	0 (0.0)	1 (0.71)	1 (0.71)	1 (0.71)	3 (2.14)	20 (14.26)
Gentamycin	3 (2.14)	1 (0.71)	23 (16.43)	0 (0.0)	0 (0.0)	1 (0.71)	0 (0.0)	2 (1.43)	20 (14.26)
Trimethoprim-sulfamethoxazole	7 (5)	1 (0.71)	54 (38.57)	1 (0.71)	3 (2.14)	3 (2.14)	1 (0.71)	6 (4.29)	53 (37.86)
Chloramphenicol	0 (0)	0 (0.0)	21 (15)	1 (0.71)	0 (0.0)	1 (0.71)	0 (0.0)	1 (0.71)	15 (10.71)
Ciprofloxacin	4 (2.86)	0 (0.0)	36 (25.71)	1 (0.71)	0 (0.0)	0 (0.0)	0 (0.0)	3 (2.14)	34 (24.29)
Ceftriaxone	4 (2.86)	0 (0.0)	31 (22.14)	1 (0.71)	0 (0.0)	1 (0.71)	0 (0.0)	2 (1.43)	30 (21.43)
Ceftazidime	4 (2.86)	0 (0.0)	26 (18.57)	1 (0.71)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.43)	24 (17.14)
Imipenem	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nalidixic acid	4 (2.86)	0 (0.0)	41 (29.29)	1 (0.71)	0 (0.0)	2 (1.43)	0 (0.0)	3 (2.14)	41 (29.29)
Nitrofurantoin	0 (0)	0 (0.0)	10 (7.14)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (5)

populations. In our study we used isolates from significant bacteriuria while in the study by Ramos *et al.*, they used urine with asymptomatic bacteriuria (Ramos *et al.*, 2012). Other studies have also demonstrated phylogenetic group B1 to be associated with commensal and less virulent strains of *E. coli* (Clermont *et al.*, 2013)(Tenaillon *et al.*, 2010). We also noticed the existence of phylogenetic groups A, C, E, F and clade I/II, which could not be detected using the triplex PCR method. This was similar with other previous studies (Iranpour *et al.*, 2015) (Clermont *et al.*, 2013)(Clermont *et al.*, 2015)(Massot *et al.*, 2016). The study also recorded 41.3% of the isolates that belonged to the unknown group; this could be due to new strains or the un-typable by PCR as explained by Clermont *et al.* (Clermont *et al.*, 2013).

This study demonstrated very high rates of resistance to commonly used antibiotics, especially ampicillin, trimethoprim/sulphamethoxazole, and ciprofloxacin. This finding was similar to that of other studies (Gupta *et al.*, 2001)(Kothari & Sagar, 2008). This could also be explained by the poor antimicrobial use policy in the country evidenced by the over the counter antimicrobial prescriptions (Mukonzo *et al.*, 2013). We demonstrated increased prevalence of ESBL producing UPEC strains (43.57%). This was similar to other studies in the region (Kateregga *et al.*, 2015)(Ampaire *et al.* 2017). This could be explained by the increased pressure on third-generation cephalosporins in the management of UTIs (Ullah *et al.* 2009) (Brosh-Nissimov *et al.*, 2018).

In this study, we did not find any carbapenemases on phenotypic and genotypic screening. This was, however, different

from other studies conducted in a similar setting (Okoche *et al.*, 2015)(Kateete *et al.*, 2016).

This study showed that group B2 isolates were the most resistant to most antimicrobials (38.57%). This was similar to other similar studies (Iranpour *et al.*, 2015)(Massot *et al.*, 2016). In addition, groups D, B1, and F were least resistant, again similar to other studies (Iranpour *et al.*, 2015)(Massot *et al.*, 2016).

In conclusion, our findings showed that group B2 (40%) were the most predominant and most resistant phylogenetic group among UPEC clinical isolates. About 9 % of *E. coli* isolates belonged to the newly described phylogroups C, E, F, and clade I. We recommend routine surveillance of antibiotic resistance patterns in the region to help clinicians make the treatment options for patients with UTIs. We recommend a longitudinal study employing whole-genome sequencing of *E. coli* strains in relation to UTI acquisition this will provide more insight on the role of Phylogenetic groups in the pathogenesis Of UTIs.

Data availability

Figshare: Supplemental files_Paul Katongole et al_v2. <https://doi.org/10.6084/m9.figshare.10006418.v1> (Katongole, 2019).

This project contains the disc-diffusion antibody sensitivity data and phylogenetic group for samples taken from each patient in this study.

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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