PHYLOGROUP PROFILING AND THE GENETIC DETERMINANTS OF EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING *Escherichia coli* ISOLATED FROM CLINICAL SAMPLES IN LAGOS, NIGERIA

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ABSTRACT

Escherichia coli (*E. coli*) is one of the most commonly isolated multi-drug resistant bacteria (MDR) responsible for a wide spectrum of human infections characterized by virulence variation and capacity to produce extended-spectrum beta-lactamase (ESBL). However, the phylo-groups that most of the MDR *E. coli* strains belong are unknown. Here, the distribution of gene determinants of ESBL producing *E. coli* strains from clinical samples in Lagos, Nigeria in relation to their phylogroups was studied. One hundred and thirteen ESBL- producing *E. coli* isolates were prospectively collected from infection sites namely wound infection (n=39), urinary tract infection (n=57), puerperal sepsis (n=6), sepsis (n=5), enteritis (n=3), and throat infection (n=3) from four specialist and tertiary hospitals in Lagos State, Nigeria. The isolates were confirmed with MALDI-TOF mass spectrometry (Microflex LT, Bruker Daltonics, Germany). Antimicrobial susceptibility testing was carried out with the VITEK 2 system card N- 232 (bioMerieux SA, France), and the interpretation guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were adopted. The *E. coli* phylogroups were classified according to the Clermont typing scheme, while ESBL genes (bla_{SHV}, bla_{TEM}, and bla_{CTX-M}) were detected by Multiplex PCR amplification. The most predominant phylogroup was B2 65 (58.0%), followed by Clades I & II 16 (14.1%), E 12 (10.6.%), F 6 (5.3%), A 3 (2.7%), C 2 (1.8%), and B1 1 (0.9%), while 8 (7.1%) strains were unclassifiable. Out of all the phylogroups, strains belonging to group B2 harboured bla_{CTX-M} 10 (15.4%), one bla_{TEM} (1.5%) and 54 (83%) of co-existing (bla_{CTX-M} and bla_{TEM}); 2 (100%) phylogroup C harboured co-existing genes (bla_{CTX-M} and bla_{TEM}) and the only isolate with phylogroup B1 harboured co-existing gene (bla_{CTX-M} and bla_{TEM}). Our results show a high prevalence of bla_{CTX-M} + bla_{TEM} co- existing among ESBL-*E. coli* strains belonging to phylogroup B2 in the studied healthcare settings. Strategies for continuous monitoring, prevention, and control of ESBL-producing *E. coli strains* to checkmate their nosocomial transmission are urgently needed in the studied hospitals.

Keywords: *E. coli*, ESBL, Phylogroup, bla_{CTX-M}, and bla_{TEM}

INTRODUCTION

Extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* (*E. coli*) infections is a public health threat because these infections are difficult to manage due to multiple antibiotic resistance, leading to the availability of only a few antibiotics, that are effective against the infections. *E. coli* strains are normal flora of the gastrointestinal tract but can also cause serious infections in humans ranging from urinary tract infections (Onanuga *et al.,* 2019), surgical site infections to sepsis globally (Adenipekun *et al.*, 2016; Nwafia *et al.*, 2019a). This situation is compounded by their resistance to the third generation cephalosporins which are commonly used to treat associated infections (Umeokonkwo *et al.*, 2019). The resultant effects of this multi-drug resistance include increased cost of

management of various infections and prolonged hospital stay in many cases (Lakoh et al., 2020). The common resistance genes associated with ESBL production and multi-drug resistance in *E. coli* are plasmid-borne and are designated as bla_{SHV}, bla_{TEM}, and bla_{CTX-M}. Carriage of these genes by ESBL producing *E. coli* at varying prevalence rates in different settings have previously been reported in Nigeria (Raji *et al.*, 2013; Hertz *et al.*, 2019; Nwafia *et al.*, 2019a), albeit their phylotypes have not been elucidated.

Moreover, *E. coli* strains have a clonal structure in their populations that has been classified by scientists into phylotypes (Clermont *et al.*, 2013; Munkhdelger *et al.*, 2017) and these phylotypes are associated with virulence such as the phylogroup B2 and D responsible for extra-intestinal

infections in humans (Clermont *et al.*, 2013) and the commensals belong to phylogroups B1 and A (Duriez *et al.*, 2001). Also, phylogroup C is close to B1, many of the formerly assigned phylogroup D strains are now re-assigned as phylogroup E, and F is a sister to B2 (Clermont *et al.*, 2013). This information is pertinent for classifying the resistant strains in our isolate collection into the global epidemiological phylogroup context to know the resistance gene determinants that are associated with a particular phylogroup. Here, we used the revisited Clermont *Escherichia coli* phylo-typing method to categorize the strains in this study (Clermont *et al.*, 2013).

Notably, multidrug-resistant infections have caused enormous problems globally especially in low and middle-income countries with a lack of functional antibiotic stewardship, indiscriminate use of antibiotics, and lack of control of antibiotics use in veterinary and agriculture contributing to the high resistance experienced due to antibiotic selection pressure. Therefore, an understanding of the transmission of ESBL genes in relations to phylogroup will further provide new knowledge on the molecular epidemiology of multi-drug resistant *E. coli* and their virulent disposition, since the information about the ESBL genes and their phylogroups among the strains isolated from the clinical specimen in Nigeria is scarce,   Here, we studied the distribution of gene determinants and phylogroup profiles of clinical ESBL-producing *E. coli* strain from four health facilities in Lagos State, Nigeria.

MATERIALS AND METHODS
Isolate Collection, Identification, and Antimicrobial Susceptibility Testing
One hundred and thirteen isolates of ESBL-producing *E. coli* from our previous study were used for this study (Olalekan *et al.*, 2020). The isolates were collected from various infection sites in humans namely wound infection (n=39), urinary tract infection (n=57), puerperal sepsis (n=6), sepsis (n=5), enteritis (n=3), and throat infection (n=3) at the Hospital Laboratory of National Orthopaedic Hospital Igbobi (33); Lagos State University Teaching Hospital Ikeja (8); Lagos University Teaching Hospital Idi-araba (67); Federal Neuro-psychiatric Hospital Yaba (3),

all in Lagos State, southwest Nigeria. The isolates were collected between July 2016 to January 2018.

Identification of the bacterial strains was done conventionally using the indole production test, citrate utilization test, and lactose fermentation properties of the strains. The isolates were later confirmed with MALDI-TOF mass spectrometry (Microflex LT, Bruker Daltonics, Germany).

The antimicrobial susceptibility profile of the isolates was carried out with the VITEK 2 system card N- 232 (bioMerieux SA, France), and the interpretation guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST version 10.0) were adopted with the use of the clinical breakpoints (https://eucast.org/clinical_breakpoints/). The ESBL status was confirmed by the VITEK 2 system.

Ethical approval was obtained from the Health Research Ethics Committee of the College of Medicine of the University of Lagos (CMUL/HREC/05/17/136).

Molecular Characterization of the ESBL Gene Determinants
DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions by adding 30 µl proteinase K and 180 µl of ATL buffer to the emulsified discrete colony and incubated at 56 °C at 900 rpm; other DNA extraction procedures were according to the manufacturer's guidelines. This is followed by amplification of the ESBL genes (bla_{SHV}, bla_{TEM}, and bla_{CTX-M}) using a multiplex PCR protocol in a 25 µl reaction containing 1.9 µl of each pair of primer, 12.5 µl of Red-Taq, 1 µl of DNA, and 0.1 µl of PCR grade water.   The amplification conditions were set at the initial denaturation step of 95 °C for 5 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 2 minutes, and one final extension step at 72 °C for 10 minutes. The sequences of the primers were bla-SHV.SE 5'atgcgttatattcgcctgtg3'; bla-SHV.AS 5'tgctttgttattcgggccaa3'; TEM-164.SE 5'tcgccgcatacactattctcagaatga3'; TEM-165.AS 5'acgctcaccggctccagatttat3'; CTX-M-U1

5'atgtgcagyaccagtaargtkatggc3'; CTX-M-U2 5'tgggtraartargtsaccagaaycagcgg3' (Monstein et al., 2007). Amplicon size was 747 bp, 445 bp, and 593 bp respectively, and amplicons were separated on a 1.8% agarose gel.

Phylotyping

The *E. coli* isolates in this study were classified into phylotypes using the revisited Clermont typing scheme as published (Clermont *et al.*, 2013) targeting the following genes *chuA, yjaA, TspE4.C2, arpA,* and *trpA* using the following corresponding primers as follows chuA 1 &2, (279bp), yjaA1&2 TspE4C2.1&2 (211bp), TspE4.C2 (152bp), ArpAgpE_F&R (301bp), trpAgpC.1&.2 (219bp) as published and the amplification products were analyzed on 3% agarose gel. The phylogroup assignment was carried out according to the laydown scheme by Clermont *et al.*(2013) and the interpretation is indicated in table 1.

Data Analysis

All the characteristics of the strains such as infection type, type of sample, gender, and age of patients with corresponding isolates, antimicrobial susceptibility profiles, resistance genes, and the phylogroups were entered into the

SPSS version 20 statistical package and descriptive analysis was performed to determine the frequencies.

RESULTS

A total of one hundred and thirteen isolates of *E. coli* from clinical specimens were studied. Out of which 56 (49.6%) were recovered from male and 57 (50.4%) from female patients. The age range was from 4 days to 79 years old with a mean age of 43 years. The type of samples was as follows: urine 57 (50.4%), wound swabs 39 (34.5%), high vaginal swab 6 (5.3%), blood culture 5(4.4%), stool 3 (2.7%), and sputum 3 (2.7%).

The antimicrobial-resistant profiles of the ESBL-producing *E. coli* strain in this study show that all isolates were resistant to piperacillin, cefotaxime, and moxifloxacin, The order of resistance to the remaining antibiotics is ceftazidime 111 (98%), aztreonam 111 (98%), cefepime 110 (97.4%), ciprofloxacin 109 (96.5%), trimethoprim /sulphamethoxazole 106 (94%), tobramycin 91 (80.5%), gentamicin 88 (80%), 46 (41%) to piperacillin/tazobactam, meropenem 13 (11.5%), imipenem 11 (9.7%) and amikacin 11 (9.7%). All the isolates were however 100% susceptible to tigecycline and colistin as presented in table 2.

Table 1: Number and Percentage of Antibiotic Resistant Strains and their Various Phylo-groups

Antibiotic resistance N (%)	A (3)	B1 (1)	B2 (65)	C (2)	E (12)	F (6)	Clade I or II (16)	unclassifiable (8)	Total (113)
Piperacillin/Tazobactam	3(100%)	0	16 (25%)	2(100%)	6 (50%)	2 (33%)	13 (81%)	4 (50%)	46(41%)
Cefotaxime	3(100%)	1(100%)	65 (100%)	2(100%)	12(100%)	6 (100%)	16 (100%)	8 (100%)	113(100%)
Ceftazidime	3(100%)	1(100%)	64 (98%)	2(100%)	12(100%)	5 (83%)	16 (100%)	8 (100%)	111(98%)
Cefepime	3(100%)	1(100%)	64 (98%)	2(100%)	12(100%)	4 (67%)	16 (100%)	8 (100%)	110 (97%)
Aztreonam	3(100%)	1(100%)	65 (100%)	2(100%)	11 (92%)	5 (83%)	16 (100%)	8 (100%)	111 (98%)
Amikacin	1 (33.3%)	0	4 (6%)	2(100%)	2 (17%)	2 (33%)	5 (31%)	0	16 (14%)
Gentamicin	1 (33.3%)	0	55 (85%)	2(100%)	6 (50%)	5 (83%)	13 (81%)	6 (75%)	88 (78%)
Tobramycin	3(100%)	0	55 (85%)	2(100%)	7 (58%)	5 (83%)	13 (81%)	6 (75%)	91(81%)
Ciprofloxacin	3(100%)	1(100%)	65 (100%)	2(100%)	11 (92%)	6 (100%)	15 (94%)	6 (75%)	109 (97%)
Moxifloxacin	3(100%)	1(100%)	65 (100%)	2(100%)	12(100%)	6 (100%)	16 (100%)	6(75%)	109 (97%)
Tigecycline	0	0	0	0	0	0	0	0	0
Trimethoprim/Sulphamethoxazole	3(100%)	1(100%)	62 (95%)	2(100%)	11 (92%)	6 (100%)	15 (94%)	6(75%)	106 (94%)

**Table 2**: Interpretation of the Phylogroup Profiles Using the Modified Extended Quadruplex Phylo-typing Method

| Phylogroups	chuA (279bp)	yjaA (211bp)	TspE4:C2 (152bp)	ArpAgpE (301bp)	trpAgpC (219bp)
A	-	-	-	+	-
B1	-	-	+	+	-
B2	+	+	-	-	-
B2	+	+	+	-	-
C	-	**+**	**-**	+	+
E	+	-	+	+	-
F	+	**-**	**-**	-	-
Clade 1 or 11	-	+	-	-	-

Key, + = Presence of the gene, - = Absence of the gene

Overall, high rates of resistance were spread across all the phylogroups as shown in table 2. None of the strains in this study was resistant to tigecycline and colistin. Only 9.7% of the isolates from all the phylogroups were resistant to amikacin and the only two strains that belonged to phylogroup C were notably resistant to amikacin.

The distribution of the resistance genes in relation to the phylogroups is presented in figure 1. The most predominant phylogroup is B2 (65=58%), followed by Clades I&II 16 (14%), E 12 (11%), F 6 (5%), A 3 (2.7%), C 2 (1.8%) and B1 (0.9%). Eight (7.1%) strain phylogroups were unclassifiable.

Out of the one hundred and thirteen strains of *E. coli* in this study, 110 (97.3%) harboured the antimicrobial-resistant gene either singly or in combination which includes bla_{CTX-M} 21 (18.6%), bla_{TEM} 1 (0.9%), bla_{CTX-M} and bla_{TEM} combination was 88 (77.9%), none of the strains harbour bla_{SHV} in this study. Phylogroup B2 harboured majority of the classes of the resistance genes, in single as bla_{CTX-M} (10=15.4%), bla_{TEM} (1=1.5%) and in combination as bla_{CTX-M} and bla_{TEM} (54=83%). Phylogroup C harboured co-existing genes (bla_{CTX-M} and bla_{TEM}) = 2 (100%) and phylogroup B1 (bla_{CTX-M} and bla_{TEM}) = 1 (100%).
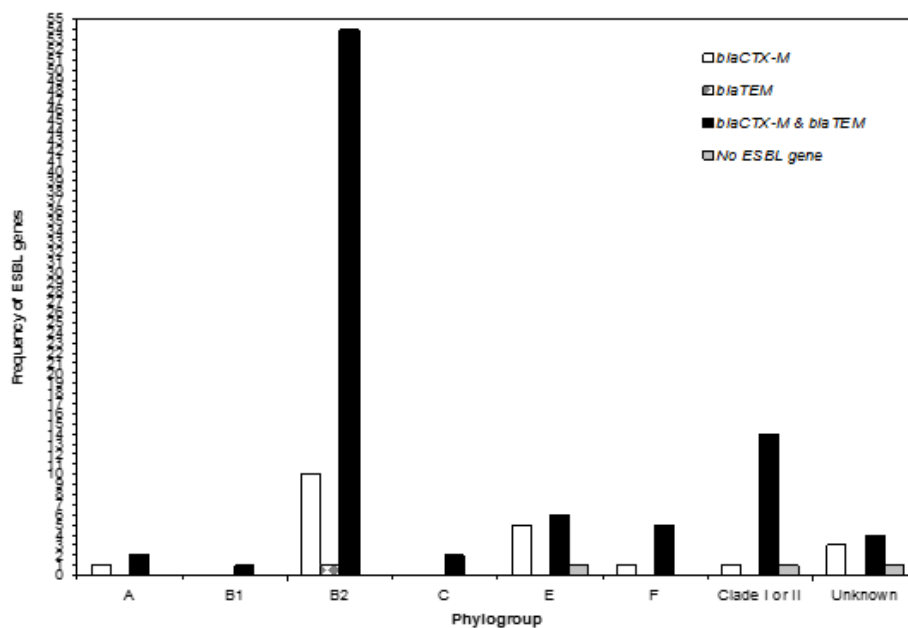
Figure 1. Distribution of ESBL Genes and their Associated Phylogroups.
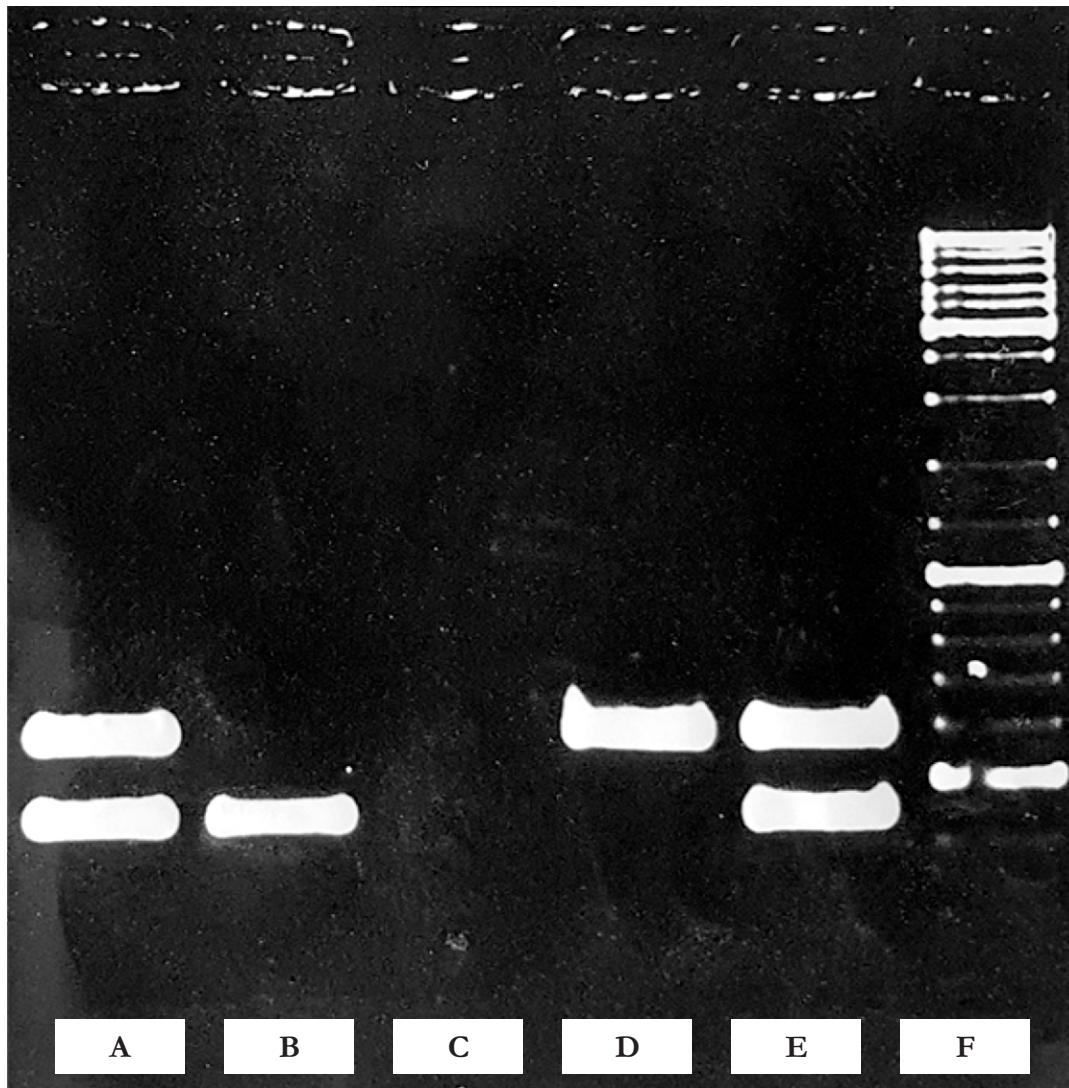
Figure 2: Electrophoretic Separation of the ESBL Genes
A = (bla_{CTX-M} & bla_{TEM}), B= bla_{TEM}, C= negative control, D= bla_{CTX-M}, E= (bla_{CTX-M} & bla_{TEM}) and F = ladder. bla_{TEM} = 445bp, bla_{CTX-M}= 593bp.

Phylogroup A harboured co-existing bla_{CTX-M} and bla_{TEM} genes 2 (66.7%) and one strain (33.3%) harboured bla_{CTX-M}; 14 (87.5%), 6 (50%), and 5 (83.3%) of isolates in the phylogroup clades I and II, group E and group F respectively harboured bla_{CTX-M} in combination with bla_{TEM} while the remaining isolates harboured only bla_{CTX-M} *gene* singly (as shown in figure1). The gel representative of the strains for ESBL is shown in figure 2.

DISCUSSION
The high mortality caused by the occurrence of ESBL- producing *E. coli* in clinical infections in our secondary and tertiary hospitals calls for the necessity to understand the genetic determinant and phylogroups of these isolates since the diversity in phylogroups is a marker for their virulence in extra intestinal infections and colonization. The presence of extended-spectrum beta-lactamase gene confers absolute resistance to the extended-spectrum cephalosporins making treatment options limited in infections caused by multi-drug resistant strains.

Phylogroup B2 is the most common in this study followed by Clade I and II and E with about 58%, 14%, and 11% respectively, majority of phylogroup B2 strains in our study were resistant to the tested third-generation cephalosporins and they harboured the ESBL genes of about 83% bla_{CTX-M} and bla_{TEM} co-existing; 15.4% bla_{CTX-M} and

1.5% *bla*$_{TEM}$ but not *bla*$_{SHV}$. Earlier reports of ESBL- producing isolates from clinical infections in Nigeria identified *bla*$_{CTX-M}$ as one of the important mechanisms that confer resistance to cephalosporins (Raji *et al.*, 2015; Nwafia *et al.*, 2019b) but most of the earlier studies did not categorize this resistance gene determinant into various phylogroups, which is a piece of important information for antimicrobial surveillance action in any community.

Only two strains in this study were classified as phylogroup C, it is worthy to note that these two strains were 100% resistant to most of the antibiotics tested including amikacin but except for susceptibility to tigecycline and colistin. Strains belonging to phylogroup C appear to have a high level of antibiotic resistance and is consistent with a study in Iran where phylogroup C was among the phylogroups in their study with multiple drug resistance but the genetic mechanisms that conferred resistance to the antibiotics including cephalosporins were not reported in their research (Iranpour *et al.*, 2015), this indicates that phylogroup C are multi-resistant to cephalosporins and penicillins and the strains possess the gene responsible for the mechanisms of resistant to this antibiotics.

Phylogroup B2 was predominant among the isolates that were classified into phylogroups. Our study is consistent with other several studies (Kõljalg *et al.*, 2014; Staji *et al.*, 2019) where phylogroup B2 has been identified mostly in extra-intestinal infections. This is because our isolates collections are from infection site and therefore belong to the pathogenic phylogroup B2. The B2 phylogroup strains are reported for its high virulence and multiple antibiotic resistance (Basu *et al.*, 2013; Ranjan *et al.*, 2017; Monroy-Pérez *et al.*, 2020). This multiple antibiotic resistance was also observed in our study which predominantly harboured co-existing *bla*$_{CTX-M}$ and *bla*$_{TEM}$ gene, a similar study carried out in Iran also reported a high prevalence of *bla*$_{CTX-M}$ among phylogroup B2 in their study (Nojoomi and Ghasemian, 2019). This is an indication of pathogenicity of phylogroup B2 strains in infections and the resistance to commonly used antibiotics in our hospitals, which can hinder management and treatment of infections caused by these strains.

Other phylogroups classified by Clermont such as phylogroups C, E, F, clades I and II were also identified in our collection of strains and harboured the *bla*$_{CTX-M}$ gene. This confirms that isolates in these phylogroups are associated with infection and are isolated from extra-intestinal sites though only a few studies have been able to confirm the environmental and virulence characteristics of strains in this phylogroups.

Only (3=2.7%) of the one hundred and thirteen isolates in this study belong to phylogroup A and only (1=0.9%) belong to phylogroup B1. Strains belonging to phylogroup B1 and group A are classified and reported as commensals (Duriez *et al.*, 2001; Chakraborty *et al.*, 2015; Mosquito *et al.*, 2015; Dadi *et al.*, 2020) and these phylogroups are few in our isolate collection, this is probably because, the strains in this study were isolated from infection site and of pathogenic nature causing infection and not colonization or commensals as it was reported earlier that these phylogroups are predominant in gut flora and are commensals (Duriez *et al.*, 2001; Chakraborty *et al.*, 2015; Mosquito *et al.*, 2015; Dadi *et al.*, 2020). In addition B1 was identified from an animal, human and food product from another recent study (Olowe *et al.*, 2019) and also the strains belonging to phylogroups B1 and A appeared to have fewer virulent determinant and therefore are commonly found to colonize a site as commensals and not a cause of infection.

About 5% of the isolates in this study belonged to phylogroup F which may be in line with the findings of Clermont *et al.* (2013) that this group is in close relationship with phylogroup B2 that made up the largest percentage of our isolates and therefore associated with infection.

About 7% of the isolates in this study could not be categorized. The reason for this is not clear but these may be newly emerging strains or novel strains whose phylotypes are yet to be discovered. Or the primers used could not bind to the DNA template, it may also be the variability in the genetic make-up of *Escherichia coli* which either gained or lost their gene(s) (mutated). About 5% of ungroupable strain collection was also reported by Clermont *et al.* (2013). Multi-locus Sequence

Typing (MLST), as well as Whole Genome Sequencing (WGS), are now a better option of grouping isolates into clonal groups especially when investigating the trend of transmission of particular isolates in a disease outbreak.

Our result confirms the high antibiotic-resistant nature of phylogroup B2 and their pathogenicity ability because they are strains that cause human infection. In addition, phylogroup class B2 harboured most of the different types of gene determinant or mechanism of extended-spectrum beta-lactam antibiotics resistance identified in this study such as co-existing bla_{CTX-M} and bla_{TEM} as well as bla_{CTX-M} occurring singly.

In conclusion, this study identified a high prevalence of the strains belonging to phylotype B2 among extra-intestinal clinical *E. coli* isolates from various hospitals in Lagos, Nigeria which harboured resistance genes to extended-spectrum cephalosporins. This information is important to drive national plans on strategies to curb and stop the spread of antibiotic resistance, especially among clinical infections in Nigerian hospital settings.

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