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Phenotypic Determination and Antimicrobial Resistance Profile of Extended Spectrum Beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Accra, Ghana

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Abstract
Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated beta lactamases commonly found in the *Enterobacteriaceae* that are capable of hydrolysing β-lactams except carbapenems and cephamycins. ESBLs confer resistance to several non-ß-lactam antibiotics. ESBL-producing organisms appear susceptible to cephalosporins *in vitro* using conventional breakpoints but ineffective *in vivo*. This work sought to determine the occurrence of ESBL in *E. coli* and *K. pneumoniae* and their antibiotic resistance profile. Four hundred *K. pneumoniae* and *E. coli* non-duplicate isolates were collected at the Central Laboratory of Korle Bu Teaching Hospital and Advent Clinical Laboratories. They were definitively identified and their minimum inhibition concentration and antibiotic sensitivity testing for 17 antibiotics were determined using Vitek 2 Compact System (bioMérieux, Marcy I'Etoile, France). The isolates were confirmed as ESBL-producing strains using the Combination Disk Synergy Method. The results indicated that 202 (50.5%) of the bacterial isolates were ESBL-producing phenotypes with high resistant to gentamicin, ciprofloxacin, tetracycline and trimethoprim/sulfamethoxazole indicating 82.2%, 79.7%, 70.8% and 97% resistant rates respectively. imipenem and amikacin were the antibiotics of choice with 99% and 94.1% susceptibility rates (MIC_{90} of ≤1µg/ml and 4µg/ml respectively). It is imperative to routinely detect ESBL-phenotypes in health facilities, implement appropriate antibiotic administration policy and infection control measures in the hospitals.
Keywords: Extended Spectrum Beta-lactamase, Antimicrobial Resistance, β-lactams, *K. pneumoniae*, *E. coli*

1.0 Introduction

Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated beta lactamases that are capable of hydrolysing β-lactams except carbapenems and cephamycins. They are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. They have been found in the *Enterobacteriaceae* and other Gram-negative bacilli. ESBL producing isolates are predominantly *Klebsiella pneumoniae* and *Escherichia coli* (Paterson and Bonomo, 2005). The known risk factors for colonization and infection with ESBL producing bacteria include admission to an intensive care unit, recent surgery, instrumentation, prolonged hospital stay and antibiotic exposure, especially to extended-spectrum beta-lactam antibiotics (Paterson and Bonomo, 2005). The use of extended-spectrum antibiotics exerts a selective pressure for emergence of ESBL-producing strains. Because ESBL enzymes are plasmid mediated, the genes encoding these enzymes are easily transferable among different bacteria. Most of these plasmids not only contain DNA encoding ESBL but also carry genes conferring resistance to several non-ß-lactam antibiotics. Consequently, most ESBL isolates are not only resistant to penicillins, cephalosporins and monobactams but also to other classes of antibiotics including aminoglycosides, fluoroquinolones, tetracyclines, nitrofurantoin and sulfamethoxazole-trimethoprim. Treatment of these multiple drug-resistant organisms has proven to be a therapeutic challenge (Todar, 2008). ESBL-producing isolates pose serious public health, financial and logistics challenges because of the limited choice of antibiotics for the treatment of infections by ESBL producing isolates. A failure to detect ESBLs and subsequent treatment with oxyimino-cephalosporins are associated with a higher risk of therapy failure (Paterson *et al.*, 2001). Other reports also indicate higher mortality rates (Kim *et al.*, 2002). This work determined phenotypic occurrence of ESBLs in *K. pneumoniae* and *E. coli* isolates, their minimal inhibition concentration and antimicrobial sensitivity profile in Accra.

2.0 Materials and Methods

2.1 Materials

Glycerol broth, blood agar and MacConkey agar were prepared according to manufacturers' guidelines. MAST ID^{TM} ESβL Detection Discs (Mast Group, UK) were used for ESBL screening and confirmation according to CLSI standards. Vitek 2 Compact System (bioMérieux, Marcy I'Etoile, France) was used to identify the isolates, determine minimum inhibition concentration of selected antibiotics and interpret the MICs according to CSLI breakpoints.

2.2 Study Sites

Lactose fermenting bacterial isolates were collected from the Central Laboratory of the Korle Bu Teaching Hospital (KBTH) and Advent Clinical Laboratories; both in the Accra Metropolis, Ghana. KBTH is the leading national referral centre for estimated 24 million people in Ghana Advent Clinical Laboratory is a private clinical laboratory with the state-of-the-art clinical diagnostic equipment located in Dzorwulu in Accra.

2.3 Sample Size

A sample size of 400 *K. pneumoniae* and *E. coli* corresponds with the standard techniques used to calculate the minimum sample size based on the expected prevalence and using appropriate levels of precision at 95% confidence level.

2.4 Inclusion Criteria

Non-duplicate pure cultures of *K. pneumoniae* and *E. coli* were used in the work.

2.5 Exclusion Criteria

All isolates not confirmed as *K. pneumoniae* and *E. coli* as well as all duplicate cultures was excluded.

2.6 Identification of Bacterial Isolates, Determination of Minimal Inhibition Concentration (MIC) and Antibiotic Sensitivity Testing

The lactose fermenting bacterial isolates stored in glycerol broth were sub-cultured on blood and MacConkey agar and incubated at 35°C for 24 hours. The pure colonies were gram-stained to confirm their Gram negative reaction. The isolates were identified as *K. pneumoniae* and *E. coli* based on their Gram stain reaction and biochemical reaction characteristics using Vitek 2 system. The Vitek 2 system uses the micro-dilution method to determine the MICs of the antibiotics. The 17 antibiotics used were ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, imipenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, tetracycline, nitrofurantoin, trimethoprim/ sulfamethoxazole. The Vitek 2 system (bioMérieux, Marcy I'Etoile, France) performs antimicrobial sensitivity testing (AST) based on kinetic analysis of growth data. The therapeutic significance of the MIC of the antimicrobials was determined using the Vitek 2 Compact system. At the end of the incubation cycle, MIC values and their interpretations (susceptible, resistant and indeterminate) were generated for each antibiotic.

2.7 Detection of ESBL Phenotype using ESBL Screening and Combined Disc Synergy Method

MAST ID^{TM} ESβL Detection Discs (Mast Group, UK) were used to screen and confirm the ESBL phenotypes. The MAST ID^{TM} ESβL Detection Discs comprise of cefpodoxime 30µg disks, cefpodoxime 30µg + clavulanic acid 10µg
disks; ceftazidime 30µg disks, ceftazidime 30µg + clavulanic acid 10µg disks and cefotaxime 30µg disks, cefotaxime 30µg + clavulanic acid 10µg disks.

Using a pure culture of the test organism, a suspension in distilled water equivalent in density to a McFarland 0.5 opacity standard was prepared. Using a sterile swab, the suspension was spread uniformly across the surface of Mueller-Hinton agar plate. Using a sterile forceps, one of each MAST ID^{TM} ESβL Detection Discs was placed onto the inoculated medium ensuring that they were evenly spaced. The plates were incubated aerobically at 35-37°C for 18 – 20 hours. The diameter of any zones of inhibition that were observed were measured and recorded. The zone of inhibition for the cefpodoxime, ceftazidime and cefotaxime was compared to that of the cefpodoxime, ceftazidime and cefotaxime plus clavulanic acid combination disks. An increase in zone diameter of ≥5mm in the presence of clavulanic acid from any or all of the sets of MAST ID^{TM} ESβL Detection Discs indicates the presence of ESBL in the test organism.

2.8 Statistical Analyses

The data from the work was collated and statistically analysed using one-way analysis of variance (ANOVA) . Results were considered significant if $p<0.05$.

3.0 Results

3.1 Bacterial Isolates

Table 1 indicates the distribution of the clinical isolates; 175 were *K. pneumoniae* and 225 were *E. coli*.

Table 1: Number of Bacterial Isolates

K. pneumoniae	*E. coli*	Total
175 (43.7%)	225 (56.3%)	400 (100%)

3.2 ESBL Producing Phenotypes

The combined disc synergy method (CDM) detected 202 (50.5%) of ESBL producers among the 400 total bacterial isolates of which 130 (74.3%) of the 175 *K. pneumoniae* and 73 (32.4%) of the 225 *E. coli* isolates were ESBL producers as shown in table 2. There was a significant difference ($p<0.05$) between the ESBL phenotypes detected in the *K. pneumoniae and E. coli* isolates.

Table 2: Occurrence of ESBL-producing Phenotypes

ESBL Detection Method	Number (%)		
	K. pneumoniae n=175	*E. coli* n=225	All Isolates n=400
CDM	129(73.7)	73(32.4)	202(50.5)

CDM: Combined Disk Synergy Method

3.3 Antimicrobial Susceptibility among ESBL-producing Isolates

The percentage antimicrobial susceptibility profile, MIC_{50} and MIC_{90} of ampicillin, amoxicillin/clavulanic, piperacillin, piperacillin/tazobactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, imipenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, tetracycline, nitrofurantoin and trimethoprim/sulfamethoxazol are indicated in table 3 and figure 1.

Table 3: Antimicrobial Susceptibility among ESBL Producers

Antimicrobial Agent ESBL-phenotypes(n=202)	No.(%) of Susceptible Isolates	Breakpoint Range S I R	MIC (µg/ml) MIC_{50}	MIC_{90}
*Ampicillin	0(0.0)	≤2 16 ≥32	***	***
Amoxicillin/Clavulanic acid	23(11.4)	≤2 16≥32	4	8
*Piperacillin	0(0.0)	≤4 32-64 ≥128	***	***
Piperacillin/Tazobactam	64(31.7)	≤4 32 ≥128	8	16
Cefoxitin	149(73.8)	≤4 16 ≥64	≤4	8
*Cefazolin	0(0.0)	≤4 16 ≥64	***	***
*Cefotaxime	0(0.0)	≤1 16 ≥64	***	***
*Ceftazidime	0(0.0)	≤1 16 ≥64	***	***
*Cefepime	0(0.0)	≤1 16 ≥64	***	***
Imipenem	200(99.0)	≤1 8 ≥16	≤1	≤1
Amikacin	190(94.1)	≤2 32 ≥64	≤2	4
Gentamicin	37(18.3)	≤1 8 ≥16	≤1	2
Ciprofloxacin	37(18.3)	≤0.25 2 ≥4	0.5	1
Norfloxacin	42(20.8)	≤0.5 8 ≥16	2	2
Tetracycline	37(18.3)	≤1 8 ≥16	≤1	4
Nitrofurantoin	73(36.1)	≤16 64 ≥512	≤16	32
Trimethoprim/Sulfamethoxazole	5(2.5)	≤20 80 ≥320	≤20	≤20

MIC_{50}: MIC at which 50% of the ESBL-phenotypes were susceptible to a particular antimicrobial agent
MIC_{90}: MIC at which 90% of the ESBL-phenotypes were susceptible to a particular antimicrobial agent
*All Penicillins and Cephalosporins are considered resistant to ESBL producers according to CLSI.

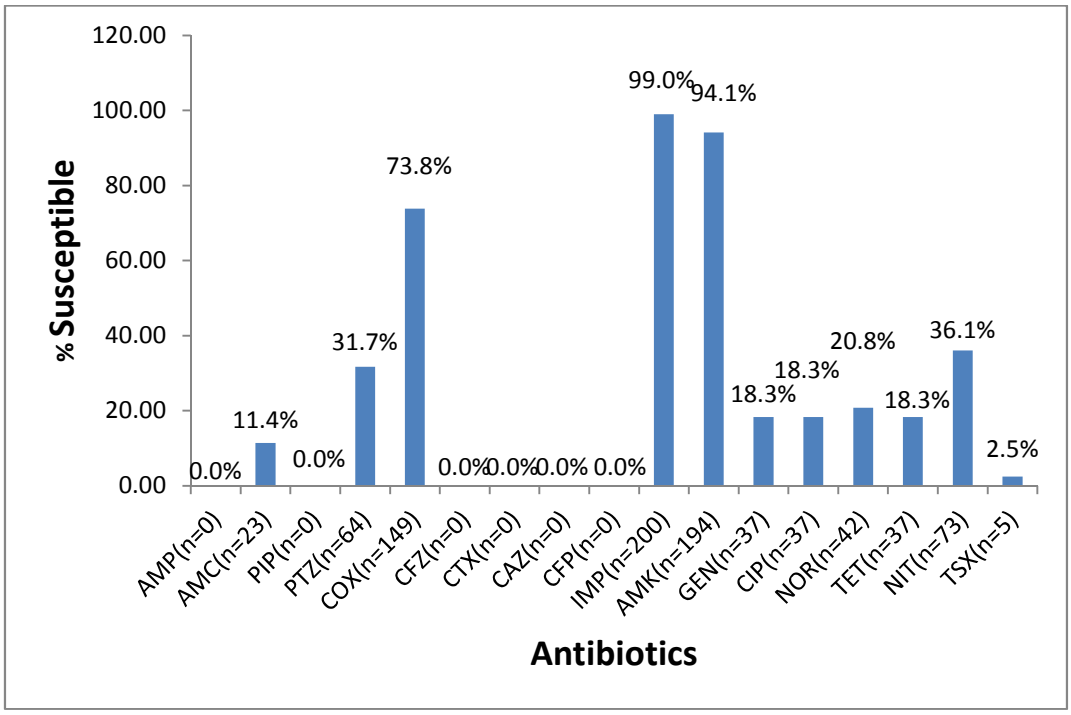

Figure 1: Antimicrobial Susceptibility among ESBL-Producers

Table 4. MIC of Penicillins and Cephalosporins in Susceptible Ranges among ESBL-Producers

Antimicrobial Agent ESBL-phenotypes (n=202)	N (%) in Susceptible Ranges	Breakpoint Range S	I	R	MIC (µg/ml) MIC_{50}	MIC_{90}
Ampicillin	0(0.0)	≤2	16	≥ 32	***	***
Piperacillin	0(0.0)	≤4	32-64	≥128	***	***
Cefazolin	5(2.5)	≤4	16	≥ 64	8	8
Cefotaxime	5(2.5)	≤1	16	≥ 64	≤1	≤1
Ceftazidime	27(13.4)	≤1	16	≥ 64	4	4
Cefepime	152(75.3)	≤1	16	≥ 64	2	8

MIC_{50}: MIC at which 50% of the ESBL-phenotypes were in the susceptible ranges to a particular antimicrobial agent

MIC_{90}: MIC at which 90% of the ESBL-phenotypes were in the susceptible ranges to a particular antimicrobial agent

3.4 Antimicrobial Resistance among ESBL-producing Isolates

The percentage antimicrobial resistance profile, MIC_{50} and MIC_{90} of ampicillin, amoxicillin/clavulanic, piperacillin, piperacillin/tazobactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, imipenem, amikacin, gentamicin,

Ciprofloxacin, norfloxacin, tetracycline, nitrofurantoin and trimethoprim/sulfamethoxazole are indicated in table 5 and figure 2.

Table 5. Antimicrobial Resistance among ESBL-Producers

Antimicrobial Agent ESBL-phenotypes (n=202)	No.(%) of Resistant Isolates	Breakpoint Range S	I	R	MIC (µg/ml) MIC_{50}	MIC_{90}
*Ampicillin	202(100)	≤2	16	≥ 32	≥ 32	≥32
Amoxicillin/Clavulanic acid	64(31.7)	≤2	16	≥ 32	≥ 32	≥32
*Piperacillin	202(100)	≤4	32-64	≥ 128	≥128	≥128
Piperacillin/Tazobactam	106(52.5)	≤4	32	≥128	≥128	≥128
*Cefazolin	202(100)	≤4	16	≥ 64	≥ 64	≥ 64
Cefoxitin	36(17.9)	≤4	16	≥ 64	32	≥ 64
*Cefotaxime	202(100)	≤1	16	≥ 64	≥ 64	≥ 64
*Ceftazidime	202(100)	≤1	16	≥ 64	16	≥64
*Cefepime	202(100)	≤1	16	≥ 64	2	32
Imipenem	2(1.0)	≤1	8	≥ 16	≤1	≤1
Amikacin	1(0.5)	≤2	32	≥ 64	≥64	≥64
Gentamicin	166(82.2)	≤1	8	≥ 16	≥16	≥16
Ciprofloxacin	161(79.7)	≤0.25	2	≥ 4	≥ 4	≥ 4
Norfloxacin	160(79.2)	≤0.5	8	≥ 16	≥16	≥16
Tetracycline	143(70.8)	≤1	8	≥ 16	≥16	≥16
Nitrofurantoin	94(46.5)	≤16	64	≥512	256	≥512
Trimethoprim/Sulfamethoxazole	196(97.0)	≤20	80	≥320	≥320	≥320

MIC_{50}: MIC at which 50% of the ESBL-phenotypes were resistant to a particular antimicrobial agent
MIC_{90}: MIC at which 90% of the ESBL-phenotypes were resistant to a particular antimicrobial agent
*All Penicillins and Cephalosporins are considered resistant to ESBL producers according to CLSI.

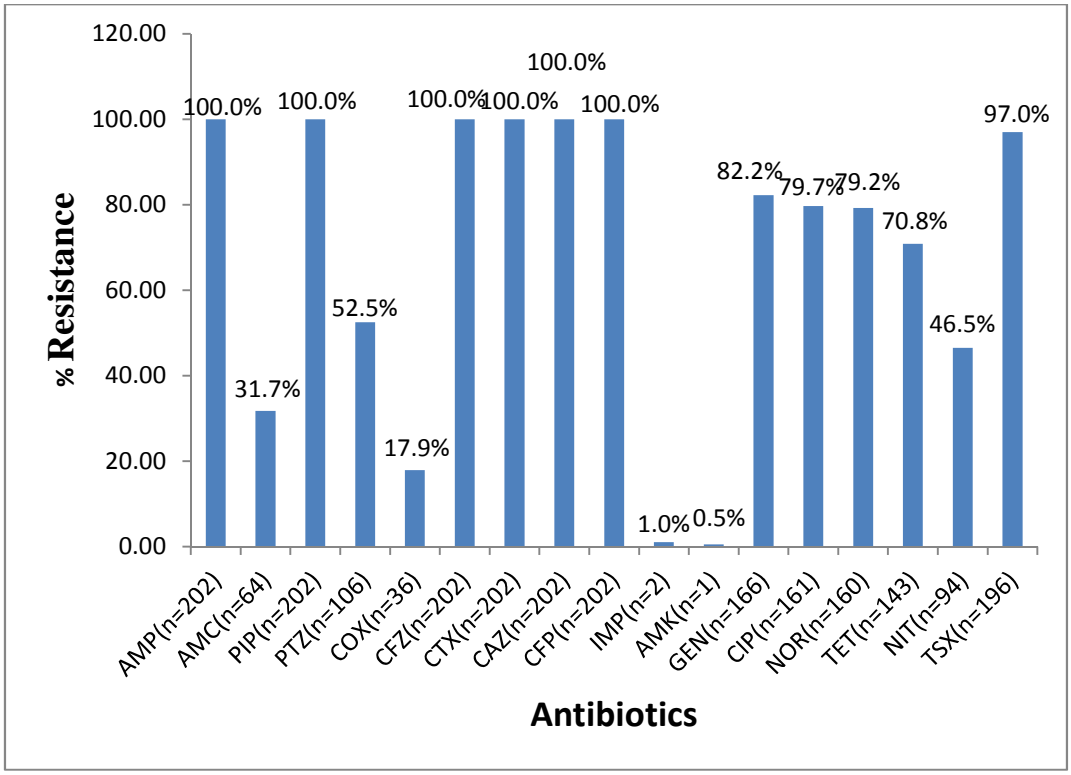

Figure 2. Antimicrobial Resistance among ESBL-Producers

4.0 Discussion

This current work sought to determine the occurrence of ESBLs in clinical isolates and their antimicrobial sensitivity profile. Of the 400 total bacterial isolates, 202 (50.5%) were ESBL producers of which 129 (73.7%) of the 175 *K. pneumoniae* and 73 (32.4%) of the 225 *E. coli* isolates were ESBL producers.

Similar work by Feglo (2013) also reported that of the 405 clinical isolates screened at Komfo Anokye Teaching Hospital, Kumasi, Ghana, 234(57.8%) were ESBL producers.

This is no different from the work published by Olysegun and colleagues in 2006 which observed 50% ESBL production rate in clinical isolates studied from northwestern Nigeria. Kesah and Odugbemi (2002) reported more than 40% ESBL production among *Enterobacteriaceae* isolates in Lagos, Nigeria. This was collaborated by Aibinu and colleagues in 2003 by reporting 42% *Enterobacter*-producing ESBL organisms in clinical isolates of from Lagos, Nigeria.

It may seem that the rate of ESBL-producing bacteria is assuming alarming rates in Ghana and West Africa. These infections may be either hospital-acquired or community-acquired ESBL-producers. The high prevalence of ESBL-producers may be attributed to prolong hospital admission and indiscriminate antibiotic exposure especially to extended-spectrum beta-lactam antibiotics used for the treatment of blood, urinary tract infections and other infectious diseases. This exerts antibiotic selective pressure for the emergence of ESBL-producing organisms in the population. Since extended spectrum beta-lactamases are plasmid mediated, the genes encoding these enzymes are easily transferable among other bacteria population thereby increasing the occurrence of ESBL-producing organisms. Nosocomial infections occur through the transmission of ESBL-producing organisms via the hands of hospital staff (Paterson and Bonomo, 2005).

However, other published works in Central, Northern and Southern Africa suggest lower occurrence rates of ESBL-producing bacteria. Thirty-one (12%) of *Enterobacteriaceae* strains isolated from 259 patients at the Yaounde Central Hospital in Cameroon were shown to be positive for **ESBL**s (**Gangoué-Piéboji et al., 2005).** In Tanzania, Blomberg and colleagues (2005) reported that 15% of 126 enterobacteria isolates studied were ESBL-producing *E. coli* and *Klebsiella* species. Bouchillon and colleagues (2004) have documented ESBL prevalence of 38.5% in Egypt. It has been reported that 36.1% of *K. pneumoniae* isolates collected in a single South African hospital in 1998 and 1999 were ESBL producers (Bell, *et al*., 2002).

It can be inferred from the results in table 2 that *K. pneumoniae* isolates produced more ESBLs than *E. coli* isolates. This is collaborated by the work of Adu-Sarkodie (2010) which reported that ESBL has been isolated from 50.3% *Klebsiella* and 49.7% *E. coli* in Komfo Anokye Teaching Hospital, Kumasi. Infections emanating from *K. pneumoniae* isolates include urinary tract infection, pneumonia, bacteremia, thrombophlebitis, cholecystitis, upper respiratory tract infection, wound infection, osteomyelitis, meningitis (Umeh and Berkowitz, 2009) and nosocomial infections (Falagas and Karageorgopoulos, 2009). Cephalosporins are recommended for the treatment of most of these Klebsiellae infections (Brooks *et al*., 2004). The increase in prevalence of these infectious diseases may lead to a corresponding indiscriminate increase in cephalosporin exposure either through prescription or over-the-counter sales. The indiscrete use of extended-spectrum cephalosporins may then lead to selective pressure resulting from genetic mutation in favour of ESBL-producing *K. pneumoniae*.

ESBLs are not active against cephamycins as demonstrated in table 3 which indicated that 73.8% of cefoxitin were susceptible to ESBL-producing isolates with MIC_{50} and MIC_{90} being ≤4µg/ml and 8µg/ml respectively. However, it has been reported that ESBL-producing strains can become resistant to cephamycins *in vivo* due to the loss of an outer membrane porin protein (Pangon *et al*, 1989).

This present work recorded high non-β-lactam resistant to ESBL-producing *E. coli* and *K. pneumoniae*. Gentamicin, ciprofloxacin, norfloxacin, tetracycline and trimethoprim/sulfamethoxazole indicated 82.2%, 79.7%, 79.2%, 70.8% and 97% resistant rates to ESBL producers. However, the resistant rates for amikacin (0.5%), imipenem (1%) and nitrofurantoin (46.5%) were slightly lower. This confirms the work of Adu-Sarkodie (2010) who indicated that the resistance prevalence of ciprofloxacin, norfloxacin, gentamicin, chloramphenicol, tetracycline, cotrimoxazole, amikacin, nitrofurantoin and imipenem were 56.73%, 79.41%, 81.43%, 90.00%, 94.23%, 96.30% 20.00%, 19.7% and 0.00% respectively.

A study by Aibinu and colleagues (2003) in hospitals in Lagos reported significant co-resistance of 75%, 89%, 63% and 100% to ciprofloxacin, streptomycin, amikacin and trimethoprim-sulfamethoxazole respectively. However, their reported resistance rated for amikacin (63%) contradicted the outcome in this present work. In contrast, Olysegun and others did not report any antibiotic resistance genes reportedly associated with ESBLs on plasmids of ESBL-producing

K. pneumoniae in Northwestern Nigeria (Olysegun *et al*., 2006). This may reflect diagnostic challenges of ESBLs in ESBL-associated antibiotic resistance prevalence.

As recommended by CLSI (2006), all penicillins and cephasporins were considered to be resistant to ESBL producers as indicated in figure 2. However, analysis of the MIC breakpoints of the cephalosporins demonstrated that 2.5%, 2.5%, 13.4% and 75.3% of cefazolin, cefotaxime, ceftazidime and cefepime showed in

vitro susceptibility to the ESBL producers as shown in table 4. This shows that in vitro susceptibility of third and fourth generation cephalosporins to ESBL producers and their subsequent use for treatment may lead to therapeutic failure if no ESBL screening tests are performed. In a randomized trial of cefepime and imipenem, clinical response for infections with ESBL-producing organisms was 100% in patients treated with imipenem but only 69% in patients treated with cefepime (Yuan, *et al*., 1998). Yu and colleagues (2002) have observed that cefepime resistance may be more frequent in strains which produce ESBLs.

Oteo and colleagues (2010) recommended the treatment of severe ESBL-producing *E. coli* infections to include the use of carbapenems, amikacin, tigecycline, and beta-lactam/beta-lactamase inhibitor combinations. For urinary tract infections, fosfomycin and nitrofurantoin could be useful. Results from this work support some aspect of this assertion especially for imipenem, amikacin and nitrofurantoin. However, amoxacillin/clavulanic acid and piperacillin/tazobactam recorded resistant rates of 31.7% (MIC_{50} and MIC_{90} is ≥32µg/ml) and 52.5% (MIC_{50} and MIC_{90} is ≥128µg/ml) respectively.

Fluoroquinolones used to be regarded as the treatment of choice for complicated urinary tract infections due to ESBL-producing organisms (Paterson and Bonomo, 2005). Unfortunately, increasing *in vitro* resistance of ESBL producers to fluoroquinolones will limit the use of these antibiotics for treating ESBL-producing infections as demonstrated in figure 2 with 79.7% and 79.2% resistant rates for ciprofloxacin and norfloxacin respectively.

5.0 Conclusion

The findings of this study suggests that there are high rates 202(50.5%) of ESBL-producing *E. coli and K. pneumonaie* in Accra and *K. pneumonaie* produces more extended spectrum beta-lactamases than *E. coli* isolates. The present work also established significant antimicrobial resistance among most of the non-β-lactam antibiotics such as ciprofloxacin, norfloxacin, gentamicin, tetracycline and trimethoprim/sulfamethoxazole. The outcome of this work recommends imipenem and amikacin as the drug of choice for treating infectious diseases caused by ESBL-producing organisms. Though nitrofurantoin may be considered in urinary tract infections, amoxicillin/clavulanic acid and piperacillin/tazobactam may not be good choice for treating infections resulting from ESBL producers.

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