Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.3, No.16, 2013

Phenotypic Characterization of AmpC beta-lactamase among Cefoxitin Resistant *Escherichia coli* and *Klebsiella pneumoniae* Isolates in Accra, Ghana

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Abstract
AmpC β-lactamases hydrolyze penicillins, cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam, and tazobactam. Strains with AmpC genes are inherently resistant to multiple agents, making the selection of an effective antibiotic difficult. This present work sought to investigate the occurrence of AmpC beta-lactamases-producing phenotypes in *E. coli* and *K. pneumoniae* and their antimicrobial sensitivity profile. Four hundred *K. pneumoniae* and *E. coli* non-duplicate isolates were collected and their antibiotic sensitivity testing for cefoxitin and other 16 antibiotics were determined using Vitek 2 Compact System (bioMérieux, Marcy I'Etoile, France). The isolates resistant to cefoxitin were confirmed as AmpC beta-lactamases-producing phenotypes with disk synergy testing (DST) using cefotaxime or ceftazidime with or without boronic acid. An increase in zone diameter of ≥5mm in the presence of boronic acid indicates the presence of AmpC beta-lactamases in the test organism. The results showed that of the 50 cefoxitin resistant isolates screened from 400 bacterial isolates, 5(10%) were AmpC beta-lactamase-producers with 60%, 60%, 60%, 80% and 100% multiply antibiotic resistance in gentamicin, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole and tetracycline respectively. Nitrofurantoin which indicated 100% susceptibility with MIC_{90} of 32µg/ml may be a therapeutic option especially for non-life-threatening urinary tract infection. Imipenem was the antibiotic of choice with 100% susceptibility rates (MIC_{90} of ≤1µg/ml). Though the insignificant ($p>0.05$) levels of AmpC beta-lactamase phenotypes may not require routine detection in health facilities, there is the need to implement evolutionary antibiotic administration policies and pragmatic infection control measures in the hospitals.
Keywords: AmpC beta-lactamase, Cefoxitin, β-lactams, *E. coli*, *K. pneumoniae*

1.0 Introduction

AmpC β-lactamases hydrolyze penicillins, cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam, and tazobactam. Many gram-negative bacilli produce a chromosomally mediated AmpC which, when hyperproduced, may cause resistance to penicillins, aztreonam, cephamycins, and cephalosporins (Thomson, 2010). The genetic determinants for AmpC β-lactamases are commonly found on the chromosomes of genera such as *Enterobacter* and *Citrobacter,* but have now transferred onto plasmids and spread to other organisms, including *E. coli* and *Klebsiella* (Heffernan *et al*., 2007). AmpC beta-lactamase-producing organisms are resistant to cefoxitin. Cefoxitin resistance can also be caused by certain carbapenemases and by decreased levels of production of outer membrane porins affecting permeability to β-lactams in both *K. pneumoniae* and *E. coli*. Nevertheless, cefoxitin resistance is said to be a discriminative parameter for the detection of AmpC-producing strains (Peter-Getzlaff *et al*., 2011). AmpC beta-lactamases are known to be inhibited by boronic acid and cloxacillin (Jacoby, 2009). Bacteria with AmpC genes are often resistant to multiple antimicrobials of different classes, making the selection of an effective antibiotic difficult. This work seeks to investigate the occurrence of AmpC beta-lactamases-producing phenotypes in *E. coli* and *K. pneumoniae* and their antimicrobial sensitivity profile.

2.0 Materials and Methods

2.1 Materials

Glycerol broth, blood agar and MacConkey agar were prepared according to manufacturers' guidelines. Cefotaxime, ceftazidime and boronic acid.antibiotics discs were used for AmpC beta-lactamase phenotype confirmation. 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 Sample Size

A sample size of 400 *K. pneumoniae* and *E. coli* were collected from the Central Laboratory of the Korle Bu Teaching Hospital (KBTH) and Advent Clinical Laboratories; both in the Accra Metropolis, Ghana. This 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.

2.3 Inclusion Criteria

Non-duplicate pure cultures of *K. pneumoniae* and *E. coli* which are resistant to cefoxitin.

2.4 Exclusion Criteria

All *K. pneumoniae and E. coli* isolates not resistant to cefoxitin.

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

The lactose fermenting isolates 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. *K. pneumoniae* and *E. coli* were identified based on their Gram stain reaction and biochemical reaction characteristics in the ID test cards wells using Vitek 2 system. The Vitek 2 system uses the micro-dilution method to determine the MICs of the antibiotics. Each AST card contains dried antibiotics with a microbiological culture medium in varying concentrations. 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.

2.6 Detection of ESBL Phenotype using AmpC beta-lactamases-producing phenotypes

AmpC beta-lactamases producers were detected by disk synergy testing (DST) using cefotaxime or ceftazidime with or without boronic acid. Using a pure culture of the test organism that were resistant to cefoxitin, 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, cefotaxime or ceftazidime with or without boronic acid 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 was measured and recorded. The zone of inhibition for the ceftazidime and cefotaxime was compared to that of the ceftazidime and cefotaxime plus boronic acid combination disks. An increase in zone diameter of ≥5mm in the presence of boronic acid indicates the presence of AmpC beta-lactamases in the test organism.

2.7 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 and AmpC beta-lactamases-Producing Phenotypes

Among the 400 bacterial isolates, 50 were resistant to cefoxitin of which 29 were *K. pneumonia* and 21 were *E. coli* as shown in table 1 and table 2. Of the 50 cefoxitin resistant isolates, 5(10%) were AmpC Beta-lactamase-producers as indicated in table 3. Only 5(1.3%) of all the 400 bacterial isolates produced AmpC Beta-lactamase as shown in table 4.

Table 1:

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

Table 2: Number of Isolates Resistant to Cefoxitin

Number (%)		
K. pneumoniae n=175	*E. coli* n=225	Total n=400
29(16.6)	21(9.3)	50(12.5)

Table 3: AmpC Beta-lactamase-producing Phenotypes among All Cefoxitin Resistant Isolates

Number (%)		
K. pneumoniae n=29	*E. coli* n=21	All Isolates n=50
2(6.9)	3(14.3)	5(10.0)

Table 4: AmpC Beta-lactamase-producing Phenotypes among All Isolates

Number (%)		
K. pneumoniae n=175	*E. coli* n=225	All Isolates n=400
2(1.1)	3(1.3)	5(1.3)

3.2 Antimicrobial Susceptibility among AmpC Beta-lactamase-producing Phenotypes

Of the 5 AmpC beta-lactamase-producing organisms, none (0.0%) was susceptible to ampicillin, amoxicillin/clavulanic acid, piperacillin, cefazolin, cefoxitin and tetracycline as shown in table 5. Of the 5 AmpC beta-lactamase-producing phenotypes, 20% of cefotaxime and ceftazidime had MICs that were susceptible and 40% of the MIC of cefepime was in the susceptible breakpoint as shown in table 5 though all cephalosporins are considered to be resistant to AmpC beta-lactamase producers. Forty percent (40%) were susceptible to piperacillin/tazobactam combination with their MIC_{50} and MIC_{90} being 8µg/ml and 16µg/ml respectively as indicated in table 5. Hundred percent (100%) of imipenem and nitrofurantoin and 60% of amikacin were susceptible to the AmpC beta-lactamase-producing organisms. Of the 5 AmpC beta-lactamase-producers, 20% were susceptible to gentamicin with their MIC_{50} and MIC_{90} being ≤1µg/ml and ≤1µg/ml respectively as indicated in table 5. Forty percent (40%) of the AmpC beta-lactamase producers were susceptible to ciprofloxacin and norfloxacin. Of the 5 AmpC beta-lactamase producers, 20% were susceptible to trimethoprim/sulfamethoxazole with their MIC_{50} and MIC_{90} being ≤20µg/ml and ≤20µg/ml respectively as indicated in table 5.

Table 5: Antimicrobial Susceptibility Profile among AmpC- Beta-lactamase Producing Phenotypes

Antimicrobial Agent	No. (%) of Susceptible Isolates	MIC (µg/ml)	
AmpC Producers (n=5)	Breakpoint Range	MIC_{50}	MIC_{90}
Ampicillin	0(0)	≤2 16 ≥32	***	***
Amoxicillin/Clavulanic acid	0(0)	≤2 16 ≥32	***	***
Piperacillin	0(0)	≤4 32-64 ≥128	***	***
Piperacillin/Tazobactam	2(40)	≤4 32 ≥128	≤4	≤4
Cefazolin	0(0)	≤4 16 ≥ 64	***	***
Cefoxitin	0(0)	≤4 16 ≥ 64	***	***
Cefotaxime	1(20)	≤1 16 ≥ 64	≤1	≤1
Ceftazidime	1(20)	≤1 16 ≥ 64	8	8
Cefepime	2(40)	≤1 16 ≥ 64	≤1	8
Imipenem	5(100)	≤1 8 ≥ 16	≤1	≤1
Amikacin	3(60)	≤2 32 ≥64	≤2	≤2
Gentamicin	1(20)	≤1 8 ≥16	≤1	≤ 1
Ciprofloxacin	2(40)	≤0.25 2 ≥ 4	≤0.25	1
Norfloxacin	2(40)	≤0.5 8 ≥16	2	2
Tetracycline	0(0)	≤1 8 ≥16	***	***
Nitrofurantoin	5(100)	≤16 64 ≥512	≤16	32
Trimethoprim/Sulfamethoxazole	1(20)	≤20 80 ≥320	≤20	≤20

MIC_{50}: MIC at which 50% of the AmpC- Beta-lactamase Producing Phenotypes were susceptible to a particular antimicrobial agent

MIC_{90}: MIC at which 90% of the AmpC- Beta-lactamase Producing Phenotypes were susceptible to a particular antimicrobial agent

3.3 Antimicrobial Resistance among AmpC Beta-lactamase-producing Phenotypes

Of the 5 AmpC beta-lactamase producing organisms, all (100%) were resistant to ampicillin, piperacillin, cefazolin, and tetracycline as shown in table 6. Forty percent (40%) and 20% of piperacillin/tazobactam and amoxacillin/clavulanic acid respectively were resistant to AmpC beta-lactamase-producing phenotypes. Sixty percent (60%) of cefotaxime and cefepime had MICs that were resistant to the 5 AmpC beta-lactamase-producing phenotypes and 40% of the MIC of ceftazidime were in the resistant breakpoint as shown in table 6. None (0%) of imipenem and nitrofurantoin and 20% of amikacin was resistant to the AmpC beta-lactamase-producing organisms. Of the 5 AmpC beta-lactamase-producers, 60% were resistant to gentamicin, ciprofloxacin and norfloxacin. Of the 5 AmpC beta-lactamase producers, 80% were resistant to trimethoprim/sulfamethoxazole with their MIC_{50} and MIC_{90} being ≥320µg/ml and ≥320µg/ml respectively as indicated in table 6.

Table 6. Antimicrobial Resistance Profile among AmpC- Beta-lactamase Producing Phenotypes

Antimicrobial Agent	No. (%) of Resistant Isolates		MIC (µg/ml)	
AmpC Producers (n=5)		Breakpoint Range	MIC_{50}	MIC_{90}
Ampicillin	5(100)	≤2 16 ≥32	≥ 32	≥ 32
Amoxicillin/Clavulanic acid	2(40)	≤2 16 ≥ 32	8	≥ 32
Piperacillin	5(100)	≤4 32-64 ≥128	≥128	≥128
Piperacillin/Tazobactam	1(20)	≤4 32 ≥128	≥128	≥128
Cefazolin	5(100)	≤4 16 ≥ 64	≥ 64	≥ 64
Cefoxitin	5(100)	≤4 16 ≥ 64	32	≥ 64
Cefotaxime	3(60)	≤1 16 ≥ 64	≥ 64	≥ 64
Ceftazidime	2(40)	≤1 16 ≥ 64	32	32
Cefepime	3(60)	≤1 16 ≥ 64	32	32
Imipenem	0(0)	≤1 8 ≥ 16	***	***
Amikacin	1(20)	≤2 32 ≥ 64	≥ 64	≥ 64
Gentamicin	3(60)	≤1 8 ≥16	≥ 16	≥ 16
Ciprofloxacin	3(60)	≤0.25 2 ≥ 4	≥ 4	≥ 4
Norfloxacin	3(60)	≤0.5 8 ≥16	≥16	≥16
Tetracycline	5(100)	≤1 8 ≥16	≥16	≥16
Nitrofurantoin	0(0)	≤16 64 ≥512	***	***
Trimethoprim/Sulfamethoxazole	4(80)	≤20 80 ≥320	≥320	≥320

MIC_{50}: MIC at which 50% of the AmpC- Beta-lactamase Producing Phenotypes were resistant to a particular antimicrobial agent

MIC_{90}: MIC at which 90% of the AmpC- Beta-lactamase Producing Phenotypes were resistant to a particular antimicrobial agent

4.0 Discussion

Fifty bacterial isolates were resistant to cefoxitin therefore potential producers of plasmid-mediated AmpC beta-lactamses (PMACBL). The occurrence of plasmid-mediated AmpC-producing strains is typically less common in most parts of the world (Jacoby, 2009). This low occurrence was reflected in this present work which recorded an insignificant ($p>0.05$) 5(10%) of the 50 cefoxitin resistant isolates as plasmid-mediated AmpC beta-lactamase producers. Of these 5, 3 were from *E. coli* and the remaining 2 were from *K. pneumoniae*. The low occurrence of AmpC beta-lactamase producers was consistent with the work of Heffernan and colleagues (2007) in New Zealand who reported that 6(18.2%) of 33 cefoxitin resistant *E. coli* isolates were AmpC beta-lactamase producers. However, in that work none of the *K. pneumoniae* isolates carried AmpC genes which deviated from this present work, though *E. coli* is deemed to be a successful producer of plasmid-mediated AmpC beta-lactamase (PMACBL). There is no published work on the occurrence of PMACBL in Accra, Ghana.

Strains with AmpC genes are inherently resistant to multiple agents, making the selection of an effective antibiotic difficult. Most cephalosporins, penicillins and β-Lactam/β-lactamase inhibitor combinations should be avoided because of *in vivo* resistance, the potential for AmpC induction or selection of high-enzyme-level mutants and documented poor clinical outcomes with ceftazidime, cefotaxime, and piperacillin-tazobactam (Jacoby, 2009). Cefepime is a poor inducer of AmpC β-lactamase which rapidly penetrates through the outer cell membrane. However, Jacoby (2009) reported that cefepime MICs increase dramatically for some AmpC producers, suggesting caution in its use, and some strains are frankly resistant. This was consistent with the present work which indicated 60% resistance of cefepime to AmpC beta-lactamase producers with MIC_{90} of 32µg/ml as shown in table 6 though 40% were susceptible with raised MIC_{90} of 8µg/ml as demonstrated in table 5. Amikacin had a good activity against AmpC beta-lactamase producers with MIC_{90} of ≤2µg/ml. Nitrofurantoin

and imipenem indicated 100% susceptibility to AmpC beta-lactamase producers with MIC_{90} of 32µg/ml and ≤1µg/ml respectively as shown in table 5. Carbapenem therapy has usually been successful when it does not lead to the emergence of carbapenem-resistant isolate associated with ACT-1 β-lactamase production and outer membrane porin loss (Jacoby, 2009). If the isolate is susceptible, nitrofurantoin therapy may be an option especially for non-life-threatening infections such as urinary tract infection.

5.0 Conclusion

The findings of this work indicated 5(10%) of AmpC beta-lactamase phenotypes among 50 cefoxitin resistant isolates screened from 400 *Escherichia coli* and *Klebsiella pneumoniae* in Accra. The AmpC beta-lactamase phenotypes exhibited multiple drug resistance of 60%, 60%, 60%, 80% and 100% to gentamicin, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole and tetracycline respectively. Nitorfurantoin which showed 100% susceptibility to AmpC beta-lactamase producers may be used in treating non-life threating urinary tract infections. Imipenem which is considered to be the antibiotic of choice indicated 100% susceptibility with MIC90 being ≤1µg/ml.

Acknowledgement

The authors appreciate the support from the management of Advent Clinical Laboratories and Central Laboratory, Korle Bu Teaching Hospital.

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