Clonal Dissemination of $bla_{TEM}$ β-lactamase Strains among Enteric Isolates in Abeokuta, Nigeria

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ABSTRACT

Recent sporadic clonal dissemination of $bla_{TEM}$ β-lactamase enteric strains was investigated among the residents and hospital patients in Abeokuta, Nigeria. The β-lactamase and ESBL production by the isolates were determined using acidometric method and phenotypic double disc test, respectively while the resistant plasmid and $bla_{TEM}$ gene was assayed by electrophoresized in agarose gel and PCR, respectively. Micro broth dilution methods were used to determine their antibiotic susceptibility. Out of 139 β-lactamase positive isolates obtained, only 38 (27.3%) expressed ESBL while 19(13.7%) had one or more $bla_{TEM}$ β-lactamase gene bands which were seen in Klebsiella oxytoca (5.6%) and Salmonella spp, Citrobacter freundii and Pseudomonas aeruginosa and Proteus mirabilis (0.7%), respectively. Epidemiological cut-off values of MIC$_{50}$ indicating = 50% of the isolates resistant at high MIC valued at $>$64 mg dL$^{-1}$ to Cefuroxime, Cefotaxime and $>$128 mg dL$^{-1}$ to Cefazidine while MIC$_{50}$ recorded at $>$90% resistant to Ampicillin, Cefuroxime, Azithromycin, Ciprofloxacin, Cefotaxime, Tetracycline at 64 and $>$128 mg dL$^{-1}$ to Gentamycin. The emergence and sporadic dissemination of mobile genetic resistant clones of TEM and AmpC beta-lactamases would become a major threat to public health sustainability without adequate antibiotic regulations in Abeokuta, Nigeria.

Key words: AmpC, $bla_{TEM}$ resistant clones, β-lactamase, antibiotic regulations

INTRODUCTION

Resistance of most enteric isolates to various antibiotics are gradually becoming a threat to global community and health-care facilities (Chaudhury and Aggarwal, 2004). Among the arrays of antibiotics of choice for treatment of enteric infections are 3rd and 4th generation cephalosporins which are now ineffective due to emergence of resistance bacteria strains producing ESBL of TEM variant (Bradford, 2001). The TEM-type ESBLs was derived from an Escherichia coli isolated from a patient in Athens, Greece, named Temoneira (hence the designation TEM) in 1965 (Jacoby, 1997). TEM-type has many genotypes, which include TEM-1 which is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin and has negligible activity against extended-spectrum cephalosporins but it is inhibited by Clavulanic acid. TEM-2 has the same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (Jacoby, 1997). The sporadic spread of this TEM is usually facilitated by the plasmid and transposon mediated β-lactamase gene which has been identified in
Klebsiella oxytoca harbouring a plasmid carrying a gene encoding ceftazidime resistance that was first isolated in Liverpool, England, in 1982 from a neonatal unit (Du Bois et al., 1995). However, the emergence of this TEM-variant to most cephalosporin is presumed to be caused by selective pressure induced by extended-spectrum cephalosporins with a number of TEM derivatives which have been found to reduce affinity for β-lactamase inhibitors (Sirot et al., 1999). In spite of this, many of them are less susceptible to the effects of β-lactamase inhibitors, by showing negligible hydrolytic activity against the extended-spectrum cephalosporins which are not considered to be ESBLs.

Interestingly, mutants TEM β-lactamases are now being recovered and could maintain the ability to hydrolyze third-generation cephalosporins which also demonstrate resistance to beta-lactamase inhibitors. These are referred to as complex mutants of TEM that is CMT-1 to 4 which are often been referred to as stably de-repressed mutants (Poirel et al., 2004). Recently, a different mechanism of resistance to expanded-spectrum cephalosporins has been recognized when Enterobacter species acquired a plasmid encoding Bush group 2be β-lactamase (Sanders and Sanders, 1977; Aibinu et al., 2003). The acquisition and spread of TEM resistance clones have developed through vertical or horizontal gene transfer or uptake of DNA from the external environment due to the death and lysis of another bacterium (Valverde et al., 2008). A single epidemic strain may carry different plasmids (carrying different ESBL genes) of genotypically non-related strains due to plasmid transfer from species to species which could be mediated by different plasmids transfer mechanism. This may imply independent evolution via the effects of antibiotic pressure or plasmid transfer from organism to organism. Transfer of genotypically related ESBLs from hospital to hospital within the city and country has been documented (Shannon et al., 1990; Hao Van et al., 2007) but not yet in Nigeria.

Therefore, the occurrence and dissemination of blaTEM β-lactamase clones was investigated in Abeokuta, Nigeria.

MATERIALS AND METHODS
Bacterial isolates: A total of 139 enteric isolates were identified as β-lactamase producing isolates according to starch-iodide paper acidimetric method described by Čudugbeni et al. (1977), from the fecal samples of consented residents and hospital patients and were further identified using modified method of Stokes and Ridgeway (1980).

Antimicrobial susceptibility testing: Susceptibility of each isolates to commonly used antibiotics was determined by standard broth-micro-dilution to the following Ampicillin (AMP) (0.5-34 µg mL⁻¹), Azithromycin (AZT) (0.5-64 µg mL⁻¹), Augmentin (AMC) (0.5-32 µg mL⁻¹), Gentamycin (GN) (0.5-64 µg mL⁻¹), Ciprofloxacin (CPX) (0.5-64 µg mL⁻¹), Cefuroxime (CFX) (1-64 µg mL⁻¹), Cefotaxime (CPX) (0.25-64), Ceftazidime (CFZ) (0.25–128 µg mL⁻¹), Tetracycline (TE) (0.25–64 µg mL⁻¹) and Imipenem (IMP) (0.25-16 µg mL⁻¹) as recommended by the NCCLS (2002).

Phenotypic ESBL detection: Enteric isolates showing a multiple resistance to more than two classes of antibiotics were tested for extended beta-lactamase enzymes (ESBL). This was performed by disc diffusion method according to the NCCLS recommendations for non-fastidious bacteria (NCCLS, 2002; Livermore and Brown, 2001) using ceftazidime (30 µg), cefotaxime (30 µg) discs alone and with the same discs in combination with clavulanic acid (10 µg) (Oxoid, UK) placed
2 cm apart. A difference of ≥5 mm between the inhibition zones of cephalothin (30 μg) plus clavulanic acid (10 μg) and cephalothin (30 μg) disc alone, was taken to indicate an ESBL producer after overnight incubation at 37°C, as recommended by NCCLS and BSAC criteria which was considered to indicate the production of ESBL. *Eschericia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 70603 were used as controls.

**Plasmid profiling:** Extraction of plasmid from each isolates positive for ESBL was done according to the alkaline lysis method developed by Bradford *et al.* (1994), using 1000 μL of the bacteria cell suspension lysed in 1% SDS, 0.2 M NaOH (pH 8.0) and precipitated in 150 μL of ice-cold 3.0 M Potassium Acetate, pH 5.5. The plasmids DNA obtained were then electrophoresed on 0.8% Agarose gel, stained with 14 μL g⁻¹ Ethidium bromide. The DNA was then photographed with Polaroid camera and viewed using UV trans-illumination. The molecular weights and distances were determined according to Kim *et al.* (2002) using standard DNA molecular weight marker of the DNA band sizes 0.12-23.1 kbp (Roche Diagnostic Gmbh).

**BlaTEM amplification and detection:** Pure colonies of identified ESBL isolates was suspended thoroughly in 1 mL DNase and RNase-free water and boiled at 55°C for 10 min. It was centrifuged at 12,000 rpm for 5 min. The DNA template obtained from the supernatant was amplified for TEM gene by Polymerase Chain Reaction (PCR), carried out in DNA thermal cycler block (NYX Technik Inc; Model ATC401, USA). The PCR mixture contains 2.5 µl 10X PCR buffer, 1.5 µl 25 mM MgCl₂, 0.5 µl of 10 mM dNTPs (dATP, dGTP, dCTP, dTTP), 0.2 µl blaTEM Forward of 2500 pmole, 0.2 µl of blaTEM Reverse of 2500 pmole, 0.2 µl Taq polymerase enzymes, 17.9 µl distilled water and 2.0 µl of each extracted chromosomal DNA to make final reaction volume of 25.0 µl. TEM gene oligonucleotide primer forward blaTEM 5’ GTA TCC GCT CAT GAG ACA ATA 3’ and reverse blaTEM 5’ TCT AAA GTA TAT ATG AGT AAA C 3’ (Poirel *et al.*, 2004) were used for the amplification. The amplification was done at in 35 cycles at 94°C for 60 sec for denaturation, annealing at 55°C for 60 sec and final extension at 72°C for 60 sec. The PCR products were electrophoresized in 1.5% agarose agar stained with ethidium bromide at 100 V along with TEM standard marker and visualized by UV light.

**Statistical methods:** The epidemiological characteristics of ESBL producers were compared using χ² tests, p value <0.05 at confidence interval of 95%. Significance of the high MIC to the occurrence of blaTEM gene was determined by Pearson χ² test while the correlation coefficient was calculated by the method of least squares.

**RESULTS**
From the Table 1; 31.6% were ESBL type of *Eschericial coli* and *Klebsiella oxytoca* while only 5.3% were *Enterobacter cloacaa* and *Salmonella* spp., respectively. Table 2 shows the epidemiological cut-off values of MIC for the ESBL isolates of MIC₉₀ indicating 50% of the isolates resistant at high MIC values >64 mg dL⁻¹ to Cefuroxime, Cefotaxime and >128 mg dL⁻¹ to Ceftazidime while MIC₉₀ representing the MIC value at which = 90% of the ESBL strains were resistant to Ampicillin, Cefuroxime, Azithromycin, Ciprofloxacin, Cefotaxime, Tetracycline at 64 and >128 mg dL⁻¹ to Gentamycin. Out of 38 ESBL-producing isolates obtained, blaTEM gene was harboured by 5.6% were *Klebsiella oxytoca* and least of 0.7% were *Salmonella* spp., *Citrobacter freundii* and *Pseudomonas aeruginosa* and Proteus mirabilis, respectively.
Table 1: Distribution of \( \beta \)-lactamase producing enteric isolates obtained from the community residents

<table>
<thead>
<tr>
<th>Enteric isolates</th>
<th>Total ESBL producer (n = 38)</th>
<th>Average resistant plasmid weight (kbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>12 (31.6)</td>
<td>11.5</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>12 (31.6)</td>
<td>10.0</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>2 (5.3)</td>
<td>8.2</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>4 (10.4)</td>
<td>7.3</td>
</tr>
<tr>
<td>Enterobacter cloaca</td>
<td>2 (5.3)</td>
<td>6.1</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>3 (7.8)</td>
<td>9.6</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>2 (5.3)</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Table 2: Distribution of \( \beta l_{TEM} \) genes among the ESBL isolates obtained and their resistance pattern to various antibiotics commonly used in this locality according to their MIC cut-off

<table>
<thead>
<tr>
<th>Enteric isolates</th>
<th>( \beta l_{TEM} ) n (%)</th>
<th>AMP (n = 35)</th>
<th>AMC (n = 12)</th>
<th>CFX (n = 38)</th>
<th>AZT (n = 15)</th>
<th>CFX (n = 61)</th>
<th>IMP (n = 35)</th>
<th>CTX (n = 13)</th>
<th>TET (n = 35)</th>
<th>CFZ (n = 11)</th>
<th>GN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>5 (3.5)</td>
<td>12</td>
<td>5</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>2</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>8 (5.6)</td>
<td>12</td>
<td>3</td>
<td>12</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>12</td>
<td>6</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>1 (0.7)</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>1 (0.7)</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter cloaca</td>
<td>2 (1.4)</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1 (0.7)</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1 (0.7)</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>MIC(_{50})</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>MIC(_{90})</td>
<td>&gt;64</td>
<td>32</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

Key: AMP-Ampicillin, AMC-Amoxicillin/Clavulanate, CFX-Cefuroxime, AZT-Azithromycin, CFX-Ciprofloxacin, IMP-Iminopen, CTX-Cefotaxime, TET-Tetracycline, CFZ-Ceftazidime, GN-Gentamycin

Fig. 1: Multiple \( \beta l_{TEM} \) genes recovered from beta-lactamase isolates on agarose gel

**DISCUSSION**

In spite of the global threat to achieving excellent therapeutic success over the resistance of \( \beta \)-lactamase inhibitors to extended-spectrum cephalosporins in many communities and hospitals all over the world, yet the mutant types are still fast spreading (Kac et al., 2008). Similar, occurrence was observed in this study where 31.6% were ESBL producer among Eschericia coli and Klebsiella oxytoca while only 5.3% were Enterobacter cloaca and Salmonella spp., respectively. These rates
were lower to other rates obtained in a sentry worldwide surveillance program report, where 45% of ESBL phenotypes of K. pneumonia strain was reported in Latin America, 23% from the Western Pacific, 23% from Europe, 8% from the United States and 5% from Canada (Winokur et al., 2001). However, very low prevalence of 1% of E. coli and K. pneumoniae strains ESBL producers were obtained in Netherlands (Bradford, 2001). From this study, greater number of ESBL isolates obtained show multidrug-resistant to more than 2 classes of antimicrobial agents with epidemiological cut-off values of MIC >64 mg mL\(^{-1}\) to Cefuroxime, Cefotaxime and >128 mg mL\(^{-1}\) to Ceftazidime while MIC >50 to Ampicillin, Cefuroxime, Azithromycin, Ciprofloxacin, Cefotaxime, Tetracycline at 64 and >128 mg dL\(^{-1}\) to Gentamycin (Poyart et al., 1998). This quite shows that a rapid emergence of resistant strains of enteric isolates which will soon become a threat to favourable treatment outcome to common enteric infections in this locality. The spread of antimicrobial resistance among bacterial pathogens to most antibiotics in this locality is fast becoming an important fatal challenge for the community and health institutions due to unguided use of over-the-counter drugs mostly antibiotics.

In recent past, there are reports of ESBL producing isolates of E. coli and K. pneumoniae in Western part of Nigeria (23) but no report of bla\(_{TEM}\) variant yet. Although, the overall frequency of bla\(_{TEM}\) reported worldwide was relatively low compare to 13.3% enteric isolate possessing bla\(_{TEM}\) gene among Klebsiella oxytoca (5.7%), Eschericia coli (8.5%) and Enterobacter cloacae (1.4%) observed in this study. This could be identified to be associated with nosocomial and community-acquired infections, that were responsible for resistant to commonly used 3rd generation cephalosporins which were genetically examined by gene profiling. In fact, this is an evidence that both bla\(_{TEM}\) gene of ESBLs has a particular propensity for community spread and it could have been already established in several areas of this township (Bradford et al., 1994). However, some of the antibiotic resistance genes usually associated with mobile elements were also associated with chromosomal transmission of resistant gene which could maintain spread TEM extended spectrum \(\beta\)-lactamases in pathogenic bacteria populations, even if patients are not treated with extended-spectrum cephalosporins, especially in most developing countries where antibiotics usage is unregulated. Multiple bands of bla\(_{TEM}\) gene among majority of the ESBL isolates could encode various bla\(_{TEM}\) variants that could cause a potential outbreak and uncontrollable dissemination of bla\(_{TEM}\) variants in this community (Fig. 1). However, increased frequency of bla\(_{TEM}\) gene that contained a pool of reserved antibiotic resistance mobile genetic element could be readily disseminated to other human pathogen which could constitute threat to human health. The application of good hygiene practices along the food chain and the prudent use of antibiotics in food animals are therefore essential. Also, constant release of resistant naked DNA digested by restriction endonucleases in a favourable condition, can allow DNA from bla\(_{TEM}\) isolate to becomes integrated into other bacteria cell which is an opportunities for cross-transmission of nosocomial pathogens via the poor hand hygiene by the food handlers, healthcare workers and moist inanimate surfaces (Poirol et al., 2004; Kac et al., 2008), thereby raising the possibility of vast dissemination of an exogenous origin of antibiotic-resistant gram-negative bacteria among the community people who become the carrier. Therefore, adequate routine surveillance of bla\(_{TEM}\) ESBL strains is highly necessary for infection control and prevention of its possible outbreak especially in this locality in Nigeria.

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