Extended spectrum β-lactamase genes in type II diabetic patients with asymptomatic bacteriuria in Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State

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ABSTRACT

Background: Antibiotic resistance has become one of the most important clinical challenges today, so also is diabetes mellitus. The immune and metabolic deficiency state in diabetic patients predisposes them to lots of nosocomial and community acquired infections which may remain asymptomatic and the causative organism may already possess antibiotic resistance genes at that level. Aim: Isolation and identification of uropathogens from type II diabetes outpatients with significant asymptomatic bacteriuria, antibiotic sensitivity profiling of the uropathogens to cephalosporins, identification of extended spectrum beta lactamase producing uropathogens and genes conferring resistance. Methods: Isolates from samples with significant bacteriuria were identified and subjected to antibiotic susceptibility test by Kirby-Bauer disk diffusion method. Multidrug resistant isolates were screened for beta lactamase production by the starch iodometric method and double disk synergy test respectively followed by Multiplex PCR for genotyping for ESBL genes. Results: Out of the 200 patient screened for significant bacteriuria, 29 (14.5%) presented with significant bacteriuria, 2 were symptomatic while 27 were asymptomatic. Prevalent uropathogens isolated were Escherichia coli (44%), Proteus mirabilis (19%) and Klebsiella pneumoniae (15%). 100% resistance was recorded for cefepime and ceftazidime, 41% for ceftriaxone, 37% for cefuroxime and 4% for imipenem. Electrophoresis of PCR products confirmed that the uropathogens carried blaCTX and blashv genes. Conclusion: Cephalosporins being used as front line agents in treating urinary tract infections in type II diabetic patients are presently being rendered useless by antibiotic resistance genes. Continuous genetic study of antimicrobial resistance in pathogens is crucial for successful antimicrobial therapy.

Key words: Asymptomatic bacteriuria, diabetes mellitus, beta lactamase, antibiotic resistance, uropathogens, cephalosporin
INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia due to defects in insulin secretion, insulin action, or both. Type 1 diabetes also known as ‘insulin-dependent diabetes or juvenile - onset diabetes’ accounts for only 5-10% of cases of diabetes. Type II diabetes also known as ‘noninsulin dependent diabetes mellitus’ or ‘adult onset diabetes’ ranges from predominant insulin resistance with relative insulin deficiency to predominantly insulin secretory defect with insulin resistance. It is the most common endocrine disease and it is fast becoming a public health problem in Africa and the world at large. It makes about 90% of the diabetic population in Nigeria. Individuals with diabetes mellitus have an increased risk of infections.

Urinary tract infection refers to both asymptomatic microbial colonization of the urinary tract and symptomatic infection with the microbial invasion and inflammation of urinary tract system. The development of urinary tract infections depends on anatomical factors, the defense mechanisms of the host, and the virulence of the infecting organism. Diabetic kidney infections and urinary tract infections remain poorly treated despite advances in recent times. Several reports have indicated changes in the prevalence of uropathogens geographically. These observed changes have serious complications as most clinicians treat patients without recourse to laboratory guidance and such treatments are usually based on known aetiological agents and susceptibilities. These observed changes may lead to ineffective antimicrobial treatments due to antibiotic resistance.

There is a need to monitor the local trends of causative agents of urinary tract infections and their resistance pattern to antimicrobials in type II diabetic patients. Molecular epidemiology methods were used in this study to track the genes conferring resistance to cephalosporins in type II diabetic patients presenting with asymptomatic bacteriuria.

METHODOLOGY

Subjects
The subjects were adult diabetic patients attending the Diabetes Outpatient Clinic of Olabisi Onabanjo University Teaching Hospital, Sagamu.

Inclusion criteria
Subjects included in the study were adult type II diabetes mellitus patients aged 18 years and above. They were patients with unknown predisposing factors to urinary tract infection and all gave their consent to partake in this study.

Exclusion criteria
Subjects excluded from this study were type 1 diabetes mellitus patients, pregnant patients, patients on immune-modulating drugs or antibiotics, patients with prostatic problems or genital growth or cancers, patients with obstructive uropathy and patients who refused to partake in the study.

Ethical approval
The human subjects involved in this study were well briefed and they gave verbal consent without bias. The Olabisi Onabanjo University Teaching Hospital Health Research Ethics Committee (OOUTH-HREC) granted the approval to proceed on the research.

Specimen collection
Freshly voided mid-stream urine were collected into sterile universal bottles and transported to the laboratory. All urine samples with significant bacteriuria were cultured within two hours of collection.

Total aerobic plate count
“Pour plate method was used to determine the bacterial load of the urine samples obtained. “Serial dilutions of the urine samples were carried out by pipetting 1 ml of the urine into 9 ml of peptone water in a sterile test tube”. One milliliter of the dilution obtained was pipeted into another test tube till a 5th test tube was reached in order to obtain countable colonies”. One milliliter of this dilution was pipetted into another test tube till a 5th test tube was reached in order to obtain countable colonies. One milliliter of the dilution obtained was pipeted into sterile 90 mm petri dish. Cooled molten CLED agar was poured into the petri dish and left to set. The plates were incubated at 35°C - 37°C for 24 hours, then the count was determined using a colony counter. Only urine samples that yielded ≥ 10^5 cfu/ml had significant bacteriuria counts.

Isolation and identification of uropathogens
A loopful of each urine specimen was seeded on Cysteine Lactose Electrolyte deficient (CLED) agar. The primary inoculum on the plate was
streaked out to ensure discrete colonies with a wire loop following standard procedures[19]. Discrete colonies were identified using morphological (Gram staining technique) and biochemical characterization.

Antibiotic susceptibility test
The antibiotic susceptibility test of the bacterial isolates was carried out on Mueller-Hinton agar using the Kirby- Bauer disk diffusion method[20] as recommended by the Clinical Laboratory Science institute.[21] The antibiotic disks used were Ceftriaxone 30µg and Cefuroxime 30µg (Himedia Lab. Pvt. Ltd. India) and Imipenem 10µg, Cefepime 30µg and Ceftazidime 30µg (Oxoid) Ciprofloxacin 5µg, ofloxacin 5µg, amoxicillin /clavulanic acid 20/10 (30µg).

A broth culture was made by inoculating the test organism in peptone water and standardizing its turbidity with a 0.5 McFarland standard. An aliquot of 0.5 McFarland standard of the broth culture was streaked on the surface of a sterile Mueller Hinton agar plate using a sterile swab stick. The antibiotic discs were placed gently and firmly on the agar plates.[22] The plates then incubated at 35°C- 37°C for 24 hours.

The diameters of the inhibition zones were measured and recorded to the nearest millimeter. “Isolates were classified as either resistant or sensitive based on the definition of the Clinical and Laboratory Standards institute”.[23] Isolates that were resistant to at least three of the antibiotics used were considered multi-drug resistant.[23] “Escherichia coli ATCC 25922 was used as control.”[23]

Beta lactamase detection
Isolates were screened for beta lactamase production by the starch iodometric method of Odugbemi.[24] Pieces of whatman No 1 filter paper cut into strips of 5x6 cm were immersed in 1% soluble starch and dried. The strips were placed in petri dishes and soaked with a solution of 100,000 units benzyl penicillin in phosphate buffered saline. With a wire loop, colonies of 18 to 24 hour old cultures grown were spread over a surface of 2-3mm. The Petri dishes were incubated at 37°C for 30 minutes, thereafter; Lugol’s iodine solution was used to flood the plate and drained off immediately. The starch paper turned black uniformly within 30 seconds of application. Colonies with decolourized zones are positive for beta-lactamase while colonies with black background were beta-lactamase negative.

Extended spectrum beta lactamase screening
Beta lactamase positive isolates (all had reduced susceptibility to 2nd and 3rd generation cephalosporins) were screened for extended beta lactamase production using the Double Disc Synergy Test Method (DDST) according to the protocol of Sorlozano et al.[25]

Genotypic analysis
Plasmid extraction
A single colony of each organism was inoculated from the nutrient slant into 5ml of Luria-Bertani broth and incubated overnight at 37°C. The lysate was prepared by transferring 2 colonies from overnight culture of each isolate on Mueller Hilton agar into Eppendorf tubes containing 100 µL of sterile water, followed by boiling at 95°C for 10 min and centrifugation at 12,000 rpm for 2 min to discard the cellular debris.[26] Plasmid DNA formed the supernatant while the precipitates are cell debris.

Multiplex PCR for bla TEM, bla CTX-M and bla SHV gene detection
The multiplex PCR was standardized to identify bla TEM, bla CTX-M and bla SHV genes simultaneously in 20 isolates. 4.0µl of a master mix supplied in 50µl mixture containing 1.25U Taq polymerase, 5x PCR Buffer, 1.5Mm MgCl2, 200µM dNTPs was pipetted into 20 different Eppendorf tubes. 12.2µl of DNase free water was added to the master mix in each of the PCR tubes followed by the addition of 0.3µl of each oligonucleotide primer (both forward and reverse primers) of bla TEM, bla CTX-M and bla SHV genes. 2µl of plasmid DNA extract from each isolate were then added to the 18µl mixture in the Eppendorf tubes and labeled accordingly.

Amplification using Techne TC-312(PCR machine) included an initial denaturation at 94°C for 30 seconds, primer annealing at 53°C for 30 seconds and then 34 cycles with denaturation at 94°C for 45 seconds, and extension at 72°C for 60 seconds per cycle for 34 cycles, the PCR products were stored at 4°C.

Electrophoresis of PCR product
Agarose gel (1%) was prepared by weighing out 1g of Agarose and dissolving in 100ml of 1xTBE (Tris-Boric Acid-EDTA buffer) with the aid of heat in a microwave. The hot gel solution was

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cooled until the glass conical flask could be touched without discomfort, 40µl of ethidium bromide was added to the cooled molten agarose gel and then poured into the tray with combs in place. The agarose gel was transferred into the electrophoresis tank containing TBE buffer. 2µl of loading buffer was added to the PCR products and then loaded into the wells. Electrophoresis was run at 100volts for 30 minutes. The ethidium bromide stained PCR products were visualized under ultraviolet light after gel electrophoresis.

**Questionnaire**
A modified WHO guided steps questionnaire was given to the patients to obtain their demographic data, biodata, diabetes history, and associated medical and surgical history.

**Statistical analysis**
Frequency of resistant and susceptibility profile was analyzed using ANOVA on SPSS version 16.0 (Statistics package for Social Sciences). P value < 0.05 was regarded as being significant.

**RESULTS**
From the 200 participants, 29 patients (19 females, 10 males; 14.5%) presented with significant bacteriuria. From the 29 patients that presented with significant bacteriuria, 27 (8 males, 19 females; 13.5%) were asymptomatic while 2 (1%) were symptomatic. 133 (66.5%) urine specimens from the patients had insignificant bacterial counts while there was no bacterial growth recorded in 38 (19%) of the urine samples. The organisms isolated from the urine samples in order of prevalence (Table 2) are *Escherichia Coli* 12 (44%), *Proteus mirabilis* 5 (19%), *Klebsiella pneumoniae* 4 (15%), *Proteus vulgaris* and *Enterococcus faecalis* 3 (11%). Table 3 illustrates the antibiotic sensitivity pattern of the uropathogens to eight antibiotics. Out of the twenty-seven (27) isolates from the asymptomatic patients with significant bacteriuria, phenotypic Starch iodometric test confirmed nineteen (19) of the isolates positive for extended spectrum beta lactamase production by the, table 4 displays this.

The genotypic detection of the extended beta lactamase producing genes confirmed the presence of bla*<sub>SHV</sub>* and bla*<sub>CTX-M</sub>* genes. Table 5 illustrates the distribution of the genes amongst the extended beta lactamase producing isolates with significant asymptomatic bacteriuria. Figure 1 displays the result of the agarose gel electrophoresis of the PCR products.

| Table 1: Primers used for bla*<sub>TEM</sub>, bla*<sub>CTX-M</sub> and bla*<sub>SHV</sub> amplification |
|---------------------------------|---------------------------------|-------------------|
| Primer target               | Sequence                        | Product size (base pairs) |
| bla*<sub>TEM</sub>             | 5’-GAGTATTCAACATTTCCGTGC-3’ (F)  | 1020bp             |
|                                | 5’-TACCAATGCTTAATCAAT-3’ (R)    |                   |
| bla*<sub>SHV</sub>             | 5’-ATGCGTTATATTCGCTGT-3’ (F)    | 400bp              |
|                                | 5’-TTAGCGGTGCCTGAC-3’ (R)       |                   |
| bla*<sub>CTX-M</sub>           | 5’-ATGTCAGGACCAGTACG-3’ (F)     | 350bp              |
|                                | 5’-TGGGTGACATTTGAC-3’ (R)       |                   |
Table 2: Prevalence of uropathogens with significant asymptomatic bacteriuria

<table>
<thead>
<tr>
<th>Organism isolated</th>
<th>Number of isolates</th>
<th>% occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>3</td>
<td>11</td>
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Table 3: Antibiotic sensitivity pattern of uropathogens to eight antibiotics

<table>
<thead>
<tr>
<th></th>
<th>CTR</th>
<th>CXM</th>
<th>IPM</th>
<th>FEP</th>
<th>CAZ</th>
<th>CIP</th>
<th>OFL</th>
<th>AMC</th>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (12)</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (4)</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (5)</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> (3)</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> (3)</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

CTR- CEFTRIAXONE, CXM- CEFUROXIME, IPM- IMIPENEM, FEP- CEFEPIME, CAZ- CEFTAZIDIME, CIP- CIPROFLOXACIN, OFL- OFLOXACIN, AMC- AMOXICILLIN CLAVULANIC ACID, S = SENSITIVE, R = RESISTANT
Table 4: Prevalence of extended spectrum beta lactamase producing uropathogens

<table>
<thead>
<tr>
<th>Uropathogens isolated.</th>
<th>Number of ESBL producers.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (12)</td>
<td>7</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (4)</td>
<td>4</td>
</tr>
<tr>
<td><em>P. mirabilis</em> (5)</td>
<td>3</td>
</tr>
<tr>
<td><em>P. vulgaris</em> (3)</td>
<td>2</td>
</tr>
<tr>
<td><em>E. faecalis</em> (3)</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5: Distribution of the ESBL genes amongst the uropathogens with significant asymptomatic bacteriuria

<table>
<thead>
<tr>
<th>Organism</th>
<th><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;</th>
<th><em>bla</em>&lt;sub&gt;SHV&lt;/sub&gt;</th>
<th><em>bla</em>&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (4)</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (6)</td>
<td>-</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>P. mirabilis</em> (3)</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>P. vulgaris</em> (2)</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>E. faecalis</em> (4)</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
DISCUSSION

Diabetes mellitus, the most common endocrine disease is fast becoming a public health issue in developing countries, including Nigeria with its ever-increasing incidence and prevalence rates. Diabetes mellitus has been known to be a predisposing factor for urinary tract infections. The world wide spread of multi drug resistant pathogens, which also limits treatment options has become another burden of public health interest.\(^{27,28}\) High concentration of glucose in the urine may create a growth medium for uropathogens and immunologic impairment leading to decline host defense system have been reported to increase the risk of asymptomatic bacteriuria.\(^{29}\) The prevalence of asymptomatic bacteriuria (13.5%) is comparable to those observed in other studies; 13.7% and 16% reported by Lyamuya and Odetoyin\(^{30,31}\) respectively. Higher values have been reported in past studies, 26.6%, 26% and 31.7% were reported by Alebiosu,\(^{32}\) Geerlings\(^{33}\) and Makuyana\(^{34}\) respectively.

The isolates recovered from the urine samples of type II diabetic mellitus patients with asymptomatic bacteriuria were the same (mostly Enterobacteriaceae) with those isolated in previous studies. Arpin \textit{et al}. reported \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} to be common causative agent in urinary tract infections, particularly amongst outpatients.\(^{35}\)

The antibiotic susceptibility profile of isolates in this study revealed a multi drug resistance pattern, which is worrisome. These results are similar to reports from other studies. This phenomenon is noticed because genes coding for resistance to different antibiotics either in the same class or different classes are often borne on the same plasmids.\(^{36}\)

The occurrence of resistance to second, third and fourth generation cephalosporins in this study necessitated a query for extended beta lactamase enzyme production. Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated rapidly evolving groups of beta lactamases capable of hydrolyzing third and fourth generation cephalosporins and aztreonam yet are inhibited by clavulanic acid,\(^{37}\) though
inhibitor resistant variants are available. The identification of the genes involved in extended beta lactamase mediated resistance is necessary for the surveillance and molecular epidemiological studies of their transmission in hospitals.\textsuperscript{34} Resistance was very high to amoxicillin-clavulanic acid in this study. This is a drug which most doctors and pharmacists recommend care freely, this could be as a result of past excellent therapeutic result achieved with the drug. In a study of prescription drug trends, Kreling \textit{et al.} reported that amoxicillin-clavulanic acid was the only antibiotic in the top 20 prescription drugs.\textsuperscript{38}

Resistance to ceftazidime and Cefepime (3rd and 4th generation cephalosporin) were 100%. Other studies too have reported this scenario.\textsuperscript{39} This scenario depicts how fast we are losing one of the best classes of antibiotics the world ever had; the beta lactam antibiotics. Shukla \textit{et al.}\textsuperscript{40} reported 72% of gram negative bacteria were resistant to all 3rd generation cephalosporins while Mojtaba and Behnaz\textsuperscript{41} reported that most resistance from their isolate were to 3rd generation cephalosporins such as cefotaxime (44%), ceftiraxone (44%) and ceftazidime (42%) while all of the isolates were susceptible to imipenem and meropenem. In this study, susceptibility to imipenem was high. Resistance to extended spectrum beta cephalosporins in this study is a well-recognized problem amongst the enterobacteriaceae.\textsuperscript{42}

Resistance to the quinolones; ciprofloxacin and ofloxacin were moderate. This pattern was also noticed by Idowu and Odelola.\textsuperscript{43} Quinolones still give a right ray of hope, but serious care must be addressed with the prescription patterns of the drugs in that class. Plasmid extraction, isolation and amplification of \textit{bla}_{TEM}, \textit{bla}_{CTX-M} and \textit{bla}_{SHV} genes by multiplex PCR (figure 2) revealed that none of the isolates were carrying TEM enzymes but they carried CTX-M and SHV enzymes, CTX-M and SHV enzymes co-existed in eight of the sixteen positive extended spectrum beta lactamase (ESBL) isolates.

The CTX-M and SHV were of the inhibitor resistant variants because they exhibited resistance to the beta lactamase inhibitor clavulanic acid. Munday \textit{et al.}\textsuperscript{44} and Valverde \textit{et al.}\textsuperscript{45} proved that CTX-M extended spectrum beta lactamases are carried by enterobacteriaceae in the community faecal flora and they are majorly responsible for urinary tract infections. Jacoby \textit{et al.}\textsuperscript{46} reported that SHV type extended spectrum beta lactamases are more frequently found in clinical isolates than any other type of extended spectrum beta lactamase.

Many organisms may produce multiple extended spectrum beta lactamase.\textsuperscript{47,48} The same organism may harbor both CTX-M- type and SHV- type ESBL or CTX-M type ESBL and Amp C- type beta lactamase which may alter the antibiotic resistance phenotype\textsuperscript{49} as experienced in this study.

In the field of infectious diseases, there is an urgent need for global approaches that can effectively and precisely study structural and functional genomics and proteomics of microbial infections. The combination of new and traditional approaches will help overcome the challenges we are facing today.

From this research work, conclusion can be drawn that \textit{bla}_{CTX-M} and \textit{bla}_{SHV} genes are present in the genome of uropathogens isolated from the urine of type II diabetes patients with asymptomatic bacteriuria at the Olabisi Onabanjo University Teaching Hospital, Sagamu. Some uropathogens possessed a combination of two or more different genes that code for several resistances to antibiotics. The observations from this study could be associated with the ease of acquisition and horizontal or vertical transmission of resistance genes borne on mobile genetic elements such as plasmids, integrons and transposons.

The emergence and spread of extended spectrum beta lactamase-producing uropathogens is worrisome, especially at the asymptomatic stage of the urinary tract infection. This means by the time the infection becomes symptomatic, the use of cephalosporins against these organisms would be a waste of time and resources.

It is recommended that more researches be conducted to proffer solutions either by way of novel antibiotics or products that can silence or switch off resistance genes in pathogenic organisms. Also, interventions such as antimicrobial stewardship should be designed to improve and measure the appropriate use of antimicrobials by promoting the selection of the optimal antimicrobial drug regimen. Infection and
antimicrobial control programs such as the MYSTIC Program (Meropenem Yearly Susceptibility Test Information Collection) should be setup and revised periodically in Nigeria. Up to date and revised information from these programs will help physicians in better antimicrobial prescriptions.

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Conflict of Interest: None declared
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