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Studies On Extended Spectrum Beta-Lactamase Producing Escherichia Coli In Men With Epididymitis Infection In A Tertiary Hospital In Enugu State, Nigeria

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Abstract
Background: *The spread of Extended Spectrum Beta-Lactamases (ESBLs) producing Escherichia coli poses a major therapeutic challenge today in the treatment of hospitalized and community-based patients. Hence, this study aimed to determine the prevalence of ESBLs producing and Multi-Drug Resistant (MDR) Escherichia coli from men attending prostate clinics at 82 Division Hospital Enugu, Nigeria.*
Materials and Methods*: A total of 50 urine samples were collected and cultured on Eosin methylene blue agar and processed by standard microbiological methods. Antimicrobial susceptibility testing was performed using Kirby-Bauer disc diffusion method on Muller-Hinton agar. Polymerase Chain Reaction was used to detect the ESBLs producing Escherichia coli specific Temoniera in Greece (TEM) genes.*
Results*: Escherichia coli isolates were highly prevalent at 26(56%) and highly resistant to most of the test agents. Multi-drug resistant Escherichia coli isolates were positive for TEM gene detection for ESBLs producing Escherichia coli.*
Conclusion*: The results from this study show there is a high prevalence of ESBLs producing Escherichia coli among men attending prostate clinic in the study area and were resistant to the available antibiotics.*
Keywords:*Extended Spectrum Beta-Lactamases, Polymerase Chain Reaction, Multi-drug resistant Escherichia coli.*

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I. Introduction

Epididymitis is an inflammation of the coiled tube (epididymis) at the back of the testicle that stores and carries sperm. Epididymitis can occur alone or in conjunction with orchitis, (inflammation of the testicles) .It can be further classified as acute (symptoms for less than 6 weeks) or chronic (symptoms for more than 3 months).Patients with epididymitis usually present with gradual onset of unilateral scrotal pain, discomfort, and tenderness in addition to palpable swelling, fever, urgency, hematuria, dysuria and other symptoms associated with lower urinary tract infections may also be present. Epididymitis may be caused by a virus or bacterium, but bacterial infection is the most common etiology. *Escherichia coli* an enteric organism is frequently the cause of epididymitis among men who have benign prostatic hyperplasia (BPH), urinary tract instrumentation, surgery, or who practice insertive anal sex. *Escherichia coli* which is part of the normal flora of humans and other animals and a member of the Enterobacteraceae family, have been known to cause a variety of human infections including urinary infection, severe abdominal pain, hemorrhagic colitis, and haemolytic-uremic syndrome which can be fatal. Resistance to a number of antibiotics have been reported in *Escherichia coli* such as tetracycline, nalidixic acid, ceftriaxone, chloramphenicol, gentamicin, ampicillin, kanamycin and sulfamethoxazole-trimethoprim,[2].Abuse and overuse of antibiotics in the clinic has resulted in the emergence of multiple antibiotic resistant bacteria strains[9]. In addition, an increase in the prevalence of multiple antibiotic resistant *E. coli* isolates has been reported worldwide. In recent decades, beta-lactams, as well as fluoroquinolones have been used as important therapeutic choices against bacterial infection. Therefore, the selective pressure resulting from their use and sometimes misuse contributes to antibiotic resistance[4,6]. One of the most important mechanisms is the plasmid-mediated production of extended-spectrum β-lactamases (ESBLs), which can hydrolyze β-lactams[13]. ESBLs is a group of enzymes that can hydrolyze penicillin and also can hydrolyze the first, second, and third generations of antibiotics, such as Cephalosporins and Aztreonam. ESBLs can be inhibited by enzyme inhibitors, which are sensitive to antibiotics, such as Cephamycin and Carbapenem. Bacteria that carry this enzyme can hydrolyze the corresponding antibiotics, leading to the failure

of some treatments. Two genetic bacteria systems, chromosomal DNA and extrachromosomal DNA, are contributory to the transfer of genes through many processes involving mutations, recombination and horizontal transfer of genes with a resultant bacterial genomic diversity. Thus, MDR-plasmids help spread resistance by horizontal gene transfer[3]. Strains of *Escherichia coli* are generally reported to have a large proportion of genomic plasmid codes responsible for resistance to antibiotics than those of the chromosome. The fact that antibiotics are not being produced to meet up with the rate at which bacteria resistant strains are evolving, there is the postulation that biotechnology might be a useful tool needed to cub the transfer of resistant genes. To this effect, Kamruzzaman *et al* (2003). demonstrated that plasmid interference could be used in creating plasmid incompatibility as a means for curing of antibiotic resistance plasmid genes. They recommended that plasmid research may be a useful tool suitable for clinical use in individuals who are found to have been colonized. Earlier, van Hal *et al* (2011). had demonstrated that the eradication of plasmids detected in the gut flora of patients was capable of preventing further complications such as sepsis that would have resulted from resistance to antibiotics in patients. It is therefore pertinent that there be a continual monitoring of resistance to antimicrobials by bacteria isolates as well as characterizing the genes responsible for such resistance.

II. Materials And Methods

Sampling

A total of fifty (50) clean-voided, mid-stream urine samples were collected from men attending '82 Division hospital, Enugu. Samples were collected from Jan to Feb 2023 and sent immediately to microbiology lab, Enugu State University of Science and Technology.

Isolation of Organisms

All the media used were prepared according to manufacturer's instructions and then autoclaved at 121^0C for 15 min. 0.1ml of urine sample was transferred on Eosin methylene blue agar and spread with a sterile inoculating loop. The plates were incubated at 37^0C for 24hr. Resultant colonies were purified by transferring to nutrient agar and incubated at 37^0C for 24hr. The characteristic isolates were aseptically isolated and characterized using established microbiological methods including colonial morphology, Gram's staining reaction, haemolytic reaction, catalase and coagulase tests. Identified *E.coli* colonies were stored on agar slants at 40^0C for further use.

Inoculum Standardization

Mac-Farland's turbidity standard was prepared by dissolving of 1ml barium chloride into 9ml of sulphuric acid. A loopful of the pure isolate was transferred into 5ml nutrient broth in a test tube and incubated at 28^0C for 24h. The culture was then adjusted to 0.5 Mac-Faland's turbidity standard.

Antibiotic Susceptibility Testing

The antibiotic susceptibility test was carried out as described by Kirby-Bauer disc diffusion method and interpreted according to the National Committee for Clinical Laboratory Standard (NCCLS). A total of 0.1ml of the isolate already matched to 0.5ml Macfarland's standard turbidity were transferred on Muller-hinton agar plates and incubated at 37^0C for 24h. ten common antimicrobial agents were used;

Ciprofloxacin (CPX) (10ug), nadilixic acid (NA) (10ug), Gentamycin (CN) (10ug), Amoxycillin (AMX) (30ug), septrin (S) (30ug), Reflacine (RD) (10ug), perfloxacin (PEF) (30ug), chloramphenicol (CH) (30ug), ofloxacin (OFX) (10ug), levofloxacin (LEV) (30ug) (Maxicare medical laboratory). The size of the area of suppressed growth (zone of inhibition) was determined by the concentration of the antibiotics present in the area, therefore, the diameter of the inhibition zones denotes, the relative susceptibility to a particular antibiotic. Inhibition zones were measured after 24h of incubation at 37^0C.

Molecular Characterization

Extraction of genomic DNA

The DNA extraction was done using the Zymo Research Mini Quick-DNA™ Fungal/Bacterial Miniprep Purification Kit, with catalogue number D6005. The extraction protocol is as follows; The cell cultures were harvested in a 1.5ml micro centrifuge tube by centrifuging for 10min at 5000×g. supernatant were discarded after centrifuging. Cells were resuspended with 200 µl of sterile nuclear free water, and transfer to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm). A total 750µlBashingBead™ Buffer was to the tube. The tube was vortexed at maximum speed for ≥ 5 minutes. The ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) was centrifuged in a Microcentrifuge at 10,000 x g for 1 minute. Up to 400 µl supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 x g for 1 minute. A total of 1,200 µl of Genomic Lysis Buffer was then added to the filtrate in the Collection Tube. Up to 800 µl of this mixture was transferred to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute. The

flow through was discarded from the Collection Tube and the previous step repeated. A total of 500 µl g-DNA Wash Buffer was added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml Microcentrifuge tube and 100 µl (35 µl minimum) DNA Elution Buffer was then added directly to the column matrix. It was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The purified DNA was used immediately in downstream applications or stored at 20 °C.

DNA Amplification and Agarose Gel Electrophoresis

The PCR reaction mixture contained 12.5 µl of 1X Master mix with standard buffer, 0.5 µl (10 µM) each of the forward and reverse primers, a total of 3 µl of the extracted DNA, and 8.5 µl of sterile nuclease-free water to make up to 25 µl of reaction volume. The polymerase chain reaction (PCR) was carried out using the one Taq Quick load 2X Master Mix with Standard Buffer (New England Biolabs, MA, U.S.A.), which is composed of; 20 mMTris-HCl, 1.8 mM MgCl2, 22 mM NH4Cl, 22 mMKCl, 0.2 mM DNTPS, 5% glycerol, 0.06% IGEPAL CA-630, 0.05% Tween 20, Xylene Cyanol FF, Tartrazine and 25 units/ml Taq DNA polymerase. This was vortexed at low speed and placed in a thermal cycler machine, with cycling parameters and primers used as below

Conventional PCR was done for Temoniera in Greece (TEM) gene detection,

The PCR products were analyzed on 1.5% Agarose gel stained with 1µg/ml of ethidium bromide following protocol described by Lee *et al.* (2012). Electrophoresis was carried out at 90 volts for 45 min and visualized/ illuminated under ultraviolet transilluminator. A 100 bp DNA ladder (New England Biolabs, USA) was used as DNA molecular weight marker.

III. Results

Occurrence of Urine Analysis

Out of 50 urine samples, 11(22%) were clear and pale and is regarded as normal. Abnormal parameters were 20(40%) cloudy and pale, 10(20%) cloudy and bloody according to the signs and symptoms of the epididymitis infection.

Cultural and Morphological Characteristics of Isolates

Out of 50 urine samples examined 26 (56%) yielded growth on Eosin methylene blue agar, colonies on EMB agar showed metallic green sheen.

Prevalence of Epididymitis Infection Amongst Men Attending Prostate Clinic.

Out of 50 patients attending prostate clinic 30(60%) patients experience swelling in the scrotum, 45(90%) experience painful urination, 45(90%) urinate frequently, 20(45%) had hematuria. 25(50%) experience fever, these are sign and symptoms of epididymitis infection.

Identification and Characterization of *Escherichia coli*

Escherichia coli is a Gram negative, rod shaped bacteria that showed varied biochemical characteristics

Antibiotic Susceptibility Test on *Escherichia coli*

Escherichia coli was highly resistant to most of the test agents. The isolates were completely resistant to amoxcyillin at 100% followed by ofloxacin, levofloxacin, perfloxacin, nalidixic acid, at 92.9%.

Agarose Gel Electrophoresis of PCR

Samples 2, 3, 4, 5, 6, 7, 8, 9, 10 to 11 were positive for TEM gene detection for ESBL producing *Escherichia coli*.

The primers target the TEM gene at 459bp ten (10) isolates expressed ESBLs genes specific sequence gene in their PCR product, which confirmed the assumption that they were strains of ESBLs producing *Escherichia coli*.

Table 1: The Characteristic Appearance of Urine Samples

Appearance	Total number analyzed	Percentage occurrence % N=50
Clear and pale	11	22%
Cloudy and pale	20	40%
Cloudy and bloody	10	20%
Clear amber	9	18%

Plate 1: Colonies of green metallic sheen characteristic of *Escherichia coli* on EMB

Table 2: Prevalence of Epididymitis Infection Amongst Men Attending Prostate Clinic.

Signs and Symptoms	No of positive (n-50)	No of positive with *E. coli* (n-26)
Swelling in the scrotum	30(60%)	20(76.9%)
Painful urination	45(90%)	15(57.7%)
Frequent urination	45(90%)	20(76.9%)
Hematuria	20(40%)	15(57.7%)
Fever	25(50%)	15(57.7%)

IV. Discussion

Escherichia coli is the most common organism causing both community as well as hospital-acquired urinary tract infection, often leading to serious secondary health issues[19,18]. *E. coli* was isolated at a high prevalence of 26 (56%) in this study (Table 2). This is in line with Sudheendra *et al*., 2017 who found *E. coli* as the most predominant species isolated from urinary tract infections in India. The study is also in accordance with the works of Yadav *et al*,[17] who found *Escherichia coli* as the predominately isolated organism found in urine samples at 82.5%. In the study, epididymitis was found at a high prevalence of 26 (56%). According to Bradford *et al*,[5] most cases of epididymitis are mostly caused by *E. coli* in the study, the signs and symptoms observed by the patients helped in diagnosis, swelling and redness in the scrotum occurred at 30 (60%); blood in the urine at 20 (40%); fever and chills at 25 (50%); dysuria 45 (90%) . (Table 2). Bradford *et al*;[5] postulated that most patients with epididymitis present with these signs and symptoms. This present study tries to make a strong association with the presence of *E. coli* with the signs and symptoms observed in the study population. It was observed that most of the patients who had these symptoms also had *E. coli* in their urine samples (Table 4.3). In this study, physical urine analysis also helped in elucidation of presence of infection with *E. coli* (Table

1). Cloudy and pale urine and bloody urine occurred at 20 (40%) and 10 (20%) respectively in the study population and these physical characteristics are also strong indicators of the presence of a urinary tract infection[7]. In this study, a high resistance pattern was observed amongst the *E. coli* isolates (Table 4). Occurrence was observed at Ciprofloxacin, Nalidixic acid, geatamycin, amoxicillin, septrin, perfloxacin, chloramphenicol, Reflacine, Ofloaxcin, Levofloxacin. Varied results were observed in the works of Eshetie *et al*,[7] where resistance to ciprofloxacin was observed at (46.5%). Similar resistance patterns were observed for amoxicillin at 77.6% in their study and 100% in the present study. Multi drug resistance was observed amongst the *E. coli* isolates in the study and is in line with the work of Teklu *et al*,[15] who observed multidrug resistance in *E. coli* to at least 3 antibiotics belonging to different antibiotics categories These multidrug resistant strains were further characterized to determine the ability of the isolates to producing ESBL. PCR assay was used to detect the presence of TEM in ESBLs producing *E. coli*. Ten (10) strains that showed positive result to ESBLs producing *E. coli* have molecular weight of ̲459bp. Using PCR, we confirmed that 99% (Fig. I) of the suspected isolates were able to produce ESBL. Kayastha *et al*,[21] and Teklu *et al*,[15] obtained varied results with ESBL producing *E. coli* occurring in their study area at 22(27.8%) and 52.2% respectively. In the study, 10 out of 11. ESBLs-producing *E. coli* (99%) were multidrug resistant (Table 5). The magnitude of MDR among *E. coli* strains in this study is fairly similar with studies in Gondar, Ethiopia, (93.5 and 87.4%)[1,7] . Bahir-Dar (93.1%)[8], Nepal (96.84%)[17] and Sierra Leone (85.7%)[12]. In this study, 10(99%) of the ESBL-producers were MDR strains, whereas only 1 (1%) of the non ESBLs- producers were MDR strains. Both the non-ESBL producing *E. coli* and ESBLs producing *E. coli* had increased resistance to most of the test agents.

A high prevalence of Multi-Drug Resistant ESBLs producing *Escherichia coli* was found among prostate patients in this study. Identification of ESBL organisms in routine treatment of infectious diseases in prostate patients can reduce unnecessary and inappropriate antimicrobial use. Tertiary care hospitals treating infectious diseases and even clinical laboratories can benefit by integrating antimicrobial susceptibility testing and ESBLs screening to combat the emergence of AMR and ESBLs. Routine screening of ESBLs producing *Escherichia coli* along with strong infection prevention strategies are recommended. Policies of rationalizing Antibiotics use should be put in place to strengthen the national resistance surveillance system in order to develop local antibiotics therapy guidelines and strengthening clinical bacteriology research and diagnostic capacity of laboratory professionals for the detection and surveillance of anti-biotic resistance should be encouraged.