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Research Article

Molecular Characterization of Multi Drug Resistance *Escherichia coli* isolated among Diabetes Mellitus Patients in Dongla State, Sudan

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Abstract

Background: Urinary tract infections (UTIs) caused by Escherichia coli have become a significant worldwide public health concern and is a common infectious disease in which level of antimicrobial resistance are alarming worldwide.

Methods: Urine samples were collected form Diabetic patients clinically diagnosed by having UTI, during the period from November 2019 to April 2020 at diabetic center in Dongla, Antibiotic susceptibility tests were performed by Kirby-Bauer disc diffusion and B-lactamases genes were detected used PCR.

Results: A total of 120 E. coli were Isolated from DM with UTI. All the isolated were shown to be resistant to Cefpodoxime (100%). The most efficient antibiotics were Colistin and imipenem (99.2% and 88.3 respectively as susceptibility rate) followed by Gentamycin (70%). High resistance rates were observed with ofloxacin (90.8%), Ciprofloxacin (77.5%), Amikacin (60.8%), ceftriaxone (58.3%) and Cefepime (51.7%). The most B-lactamase genes isolates were blaCTX-M -1 gene (64%) followed by blaCMY-G2 (55.8%), blaSHV gene (34.2%), blaNDM gene (10.8%), blaOXA-48 gene (5.8%), blaVIM gene (3.3%), blaKPC gene (2.5%) and blaIMP were not detected in any of the tested isolates.

Conclusions: Urinary tract infection due to E. coli may be difficult to treat empirically due to high resistance to commonly used antibiotics. Continuous surveillance of multidrug resistant organisms and patterns of drug resistance are needed to prevent treatment failure and reduce selective pressure.

Keywords: UTI, diabetes mellitus, E. coli, Multidrug-resistant, B-lactamase genes, Sudan

Introduction

Diabetes mellitus is a common endocrine disorder in which there is an insufficiency of, or resistance to, the hormone insulin, which regulates blood glucose levels. It was recorded in 2019 that worldwide 463 million people had diabetes, 90% of which was type II DM. This number is estimated to rise to 552 million in 2030 [1]. The chronic hyperglycemia in diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels [2]. DM has long been a predisposing factor for urinary tract infection (UTI) [3]. These factors include weak host immune systems with impaired neutrophil function, depressed T-cell mediated immune response, decreased production of prostaglandin E, thromboxane B2, leukotriene B44 and depressed antioxidant systems [4,5] Worldwide, UTIs' prevalence was estimated to be around 150 million persons per year [6]. Urinary tract infection can be roughly classified based on anatomy as upper UTI and lower UTI, or both. UTI can be symptomatic or asymptomatic but is defined as presence of bacteriuria with a quantitative count of more than or equal to 105 colony forming unit of bacteria per milliliter [7]. The commonest pathogenic organism isolated in UTI is E. coli followed by K. pneumoniae, Staphylococcus, Proteus, Pseudomonas, Enterococcus, and Enterobacter [8-12]. Escherichia coli is the most common causative agent of UTIs

in both DM and non-DM patients [11]. Uropathogenic E. coli (UPEC) possesses a variety of pathogenicity determinants that make colonization of the urinary tract possible. These determinants include fimbrial (P, S/F1C and type 1 fimbriae) and non-fimbrial adhesins that mediate bacterial adherence to epithelial cells, siderophores (iron-acquisition systems), secreted toxins (haemolysin and cytotoxic necrotizing factor 1) and capsule forming polysaccharides for immune evasion [13-15]. Phylogenetic analysis classifies E. coli strains into four main groups (A, B1, B2 and D). Groups B2 and D are mainly associated with E. coli strains causing extraintestinal infections, whilst groups A and B1 are associated with commensal strains [16]. Worldwide, there is great concern due to the high rates of resistance to antimicrobials used in the treatment of infections caused by E. coli, particularly in developing countries. Frequent prescription of antibiotics, including the ones with broad-spectrum, may result in development of antibiotic-resistant urinary pathogens. Since patients with DM are more prone to have resistant pathogens, they inevitably require longer and more potent antimicrobial treatment [17]. Therefore, improved control of glycaemia in diabetics may help in controlling the UTIs. Accurate screening for UTI in diabetic patients is also critical to enable the appropriate treatment, avoiding related complications. In this study we aimed to assess the prevalence of E. coli and pattern of the antimicrobial drugs susceptibility by phenotyping and genotyping method.

Material and Methods

Study design

A cross-sectional study was carrying out during the period from August 2019 to April 2020 at diabetic center in Dongla and Neelain university. The study includes patients clinically diagnosed by having one or more of the following symptoms: dysuria, frequency, urgency, suprapubic discomfort, or flank pain. Non-diabetic and pregnancy were excluded from the study. A total of 120 E. coli isolated form Diabetic patients (45 males and 75 females) with age group ranged from 10 to 80 years old. All patients were informed of the purpose of the study and their consent or that of their care provider was obtained before urine samples were collected.

Sample collection and Processing

Each patient was asked to collect approximately 10-20 ml of midstream urine into a sterile urine container. after giving proper instructions to avoid contamination and samples were processed in the laboratory within 2 hours of collection. None of the patients admitted to consuming antibiotics during the 2 weeks prior to urine sample collection.

Data collection

A structured questionnaire and referring to the patient clinical sheet were being used; demographic data and other Data (clinical symptoms, previous antibiotic, duration of antibiotic used). verbal consent was obtained from each patient enrolled in this study.

Isolation and identification of Escherichia Coli using Biochemical tests and selective medium

Urine cultures were performed using semi-quantitative technique whereby urine samples were inoculated on cystein-Lactose electrolyte deficient (CLED) medium plates with a calibrated loop (0.001ml) and incubated at 37°C for 18-24 hours. Urine culture reports that exhibited colony forming units (CFUs) more than 10^5/ml of voided urine were considered significant [18]. Isolated colonies from significant plates were identified and differentiated from related organisms using standard conventional biochemical tests (Kligler Iron agar: slant /Acid, butt/ Acid, H2S production / -, Gas / +; Motility test / motile; Indole /+, Urease / - ; Citrate /-) according to [19].

Antimicrobial susceptibility testing

Antimicrobial sensitivity testing of all isolates was performed on diagnostic sensitivity test plates according to the Kirby-Bauer method [20] following the definition of the Committee of Clinical Laboratory International Standards [21]. Bacterial inoculums were prepared by suspending the freshly grown bacteria in 5mL sterile saline. A sterile cotton swab was used to streak the surface of Mueller Hinton agar plates. Filter paper disks containing a designated concentration of the antimicrobial drugs were obtained from Hi-Media Laboratories in the following concentrations: Amikacin (30µg), Gentamycin (10µg), Cefotaxime (30µg), ceftriaxone (30µg), Meropenem (10µg) ciprofloxacin (5µg), ofloxacin (5µg), colistin (10µg), Cefepime (30µg). The diameters of zone of inhibition were interpreted according to CLSI standards. Media and disks were tested for quality control with E. coli standard strain.

Molecular detection

DNA for molecular detection was extracted after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance (CRL, 2009). Briefly, a few colonies taken from fresh culture medium and transferred to phosphate buffered saline (pH 7.3). The suspension was then heated at 100°C for 15 minutes. Boiled suspension was transferred directly on ice, this was followed by vortexing and the suspension was then centrifuged at 12000 rpm for 5 minutes to sediment the debris, the clear supernatant was used as template DNA in PCR method.

The universal primers were designed for b-lactamase genes, including ESBL genes (^{bla}SHV and ^{bla}CTX-M-1), AmpC genes (^{bla}C-MY-G2), carbapenemases genes (^{bla}KPC, ^{bla}IMP, ^{bla}VIM, ^{bla}NDM and ^{bla}-OXA-48). Details for the primers were shown in (Table 1). PCR was performed and the test was carried out in total volume of 25 µl, containing 5 µl master mix (Solis Bio dyne master mix), 2 µl of primer, 5 µl of DNA and 13 µl of distilled water. The PCR was performed for 35 cycles (initial denaturation at 95 oC for 5 min, denaturation at 95 oC for 50 s, annealing at 56 oC for 40 s or 60°C for 45, elongation at

72 oC for 1 min for 35 cycles and final extension at 72 oC for 5 min). 5 µl of the PCR product was analyzed using 1% or 1.5% Agarose gel electrophoresis and stained with 0.15% Ethidium bromide and the product was visualized using UV gel documentation [22,23].

Table 1: Primers used for PCR amplification of resistance genes.

Gene	Sequence (5' – '3)	Annealing Temp(oC)	Fragment(bp)
blaSHV	F: ATGCGTTATATTCGCCTGTG	56	896
	R: AGATAAATCACCACAATGCGC		
blaCTX-M-1	F: CCGTTTCCGCTATTACAAACCGTTG	56	944
	R: GGCCCATGGTTAAAAAATCACTGC		
blaCMY-G2	F: GGTCTGGCCCATGCAGGTGA	56	963
	R: GGTCGAGCCGGTCTTGTTGA		
blaOXA-48	F: TTGGTGGCATCGATTATCGG	56	743
	R: GAGCACTTCTTTTGTGATGGC		
blaKPC	F: TGTCACTGTATCGCCGTC	60	900
	R: CTCAGTGCTCTACAGAAAACC		
blaIMP	F: GAAGGCGTTTATGTTCATAC	60	587
	R: GTACGTTTCAAGAGTGATGC		
blaVIM	F: GTTTGGTCGCATATCGCAAC	60	389
	R: AATGCGCAGCACCAGGATAG		
blaNDM	F: GCAGCTTGTCGGCCATGCGGGC	60	782
	R: GGTCGCGAAGCTGAGCACCGCAT		

Data analysis

Statistical analysis was done by using Statistical Package for Social Science program (version 20).

Results

A total of 120 isolates were identified as E. coli from DM with UTI (45 males and 75 females, 6.7% had type 1 and 93.3% had type II DM with age group ranged from 10 to 80 years old), by routine phenotypic methods including Gram's staining, colony morphology and standard conventional biochemical tests. About 25 % and 35% of the UTI positive patients were in age between 40-50 and 50-60 years, respectively (Table 2). This result indicated that the emergence of UTI raised with the increase in age of the patient. E. coli strains showed differences in susceptibility and resistance patterns to the antimicrobials tested. All of the isolated uropathogenic E. coli were shown to be resistant to Cefpodoxime (100%). The most efficient antibiotics were Colistin and imipenem (99.2% and 88.3 respectively as susceptibility rate) followed by Gentamycin (70%). High resistance rates were observed with ofloxacin (90.8%), Ciprofloxacin (77.5%), Amikacin (60.8%) and ceftriaxone (58.3%). Resistance to Cefepime (51.7%) was a high rate of intermediate resistance (Table 3). Regarding the MDR isolates, all of E. coli isolates collected for this study showed resistance to four or more antibiotics.

Table 2: Demographic characteristics of the study participants.

	Male	Female	Total
Type of DM			
Type 1	4	4	8
Type 2	40	72	112
Duration of DM (y)			
0 – 5	16	30	46
5 – 10	13	28	41
10 – 15	7	9	16
15 – 20	5	2	7
20 – 25	3	4	7
Age group (y)			
10 – 20	4	4	8
20 – 30	0	0	0
30 – 40	1	10	11

40 – 50	11	19	30
50 – 60	15	27	42
60 – 70	8	14	22
70 – 80	6	1	7
Drug used			
Insulin	14	27	41
Metformin	30	49	79

Table 3: Antibiotic sensitivity/resistance pattern of isolated E. coli from DM UTI patients.

Antibiotics	Frequency (%)	
	Sensitive	Resistance
Amikacin	47(39.2)	73(60.8)
Gentamycin	84(70)	36(30)
Cefepime	58(48.3)	62(51.7)
Cefpodoxime	0(0)	120(100)
ceftriaxone	50(41.7)	70(58.3)
Ciprofloxacin	27(22.5)	93(77.5)
Ofloxacin	11(9.2)	109(90.8)
Meropenem	106(88.3)	14(11.7)
Colistin	119(99.2)	1(0.8)

By applying PCR method among the 120 E. coli isolates were tested for the production of B-lactamase genes, including ESBL genes (^{bla}SHV and ^{bla}CTX-M-1), AmpC genes (^{bla}CMY-G2), carbapenemases genes (^{bla}KPC, ^{bla}IMP, ^{bla}VIM, ^{bla}NDM and ^{bla}OXA-48) (Figure 1-4). The most B-lactamase genes isolates were ^{bla}CTX-M -1 gene (64%) followed by ^{bla}CMY-G2 (55.8%), ^{bla}SHV gene (34.2%), ^{bla}NDM gene (10.8%), ^{bla}OXA-48 gene (5.8%), ^{bla}VIM gene (3.3%), ^{bla}KPC gene (2.5%) and ^{bla}IMP were not detected in any of the tested isolates (Table 4).

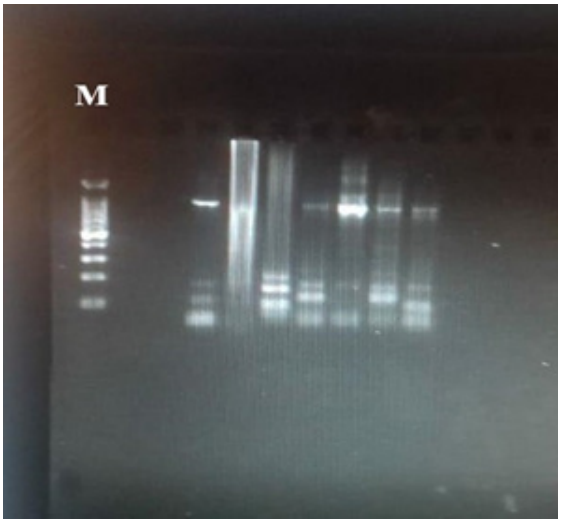

Figure 1: ^{bla}CMY-G2, ^{bla}CTX-M-1, ^{bla}KPC genes DNA results (963bp, 944bp and 900bp respectively) on 1% agarose gel. Lane M shows 100 bp DNA marker, lane 1 shows negative control, lanes 3 show positive results for ^{bla}CMY-G2, lanes 6 and 7 show positive results for ^{bla}CTX-M-1, lanes 8 and 9 show positive results for blaKPC, lane 2,4,5,10,11,12 shows negative results.

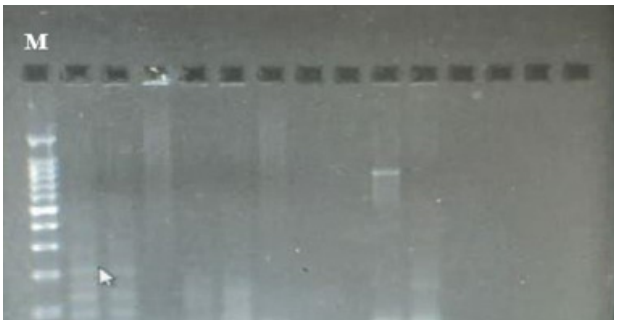

Figure 2: ^{bla}SHV gene DNA results (896bp) on 1% agarose gel. Lane M shows 100 bp DNA marker, lane 1 shows negative control, lane 9 shows positive results, lanes 2,3,4,5,6,7,8,10,11,12,13 and 14 show negative results.

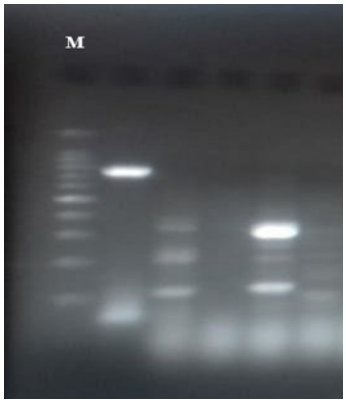

Figure 3: ^{bla}OXA-48 and ^{bla}VIM DNA results (743bp and 389bp respectively) on 1.5% agarose gel. Lane M shows 100 bp DNA marker, lane 1 shows show positive results for ^{bla}OXA-48. lane 2 and 4 shows positive results for blaVIM. lanes 2 and 5 show negative results.

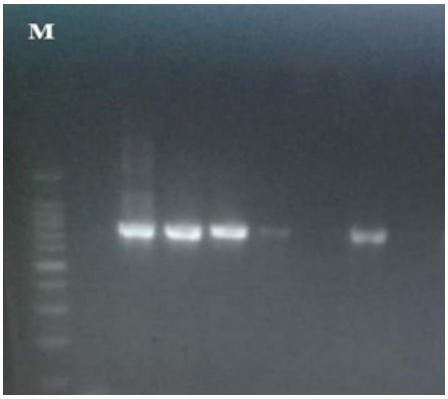

Figure 4: blaNDM DNA results (782bp) on 1.5% agarose gel. Lane M shows 100 bp DNA marker, lane 1 shows negative control, lanes 2,3,4,5, and 7 show positive results, lanes 6 and 8 show negative results.

Table 4: The distribution of β-lactamase genes among isolated E. coli.

Gene	Frequency (%)	
	Positive	Negative
blaCTX-M-1	86(64.2)	34(35.8)
blaSHV	41(34.2)	79(65.8)
blaCMY-G2	67(55.8)	53(44.2)
blaKPC	3(2.5)	117(97.5)
bla NDM	13(10.8)	107(89.2)
blaVIM	4(3.3)	116(96.7)
blaIMP	0(0)	120(100)
blaOXA-48	7(5.8)	113(94.2)

Discussion

Urinary tract infections (UTIs) are the most common human infectious disease affecting the bladder, kidneys and urinary tracts [24]. Kidney stones, diabetes, weak immune system can increase the risk of UTIs [25]. Patients with significant bacteriuria have at least two symptoms referable to the urinary tract infection (dysuria, urgency, frequency, incontinence, suprapubic pain, flank pain or costovertebral angle tenderness, fever (temp ≥ 38°C) and chills are said to be symptomatic. Complications of UTI include urosepsis, renal impairment, and renal abscess [26,27]. In the present study all urine sample (100%) revealed significant growth. the most isolated agents from urinary tract infections vary, almost all of them are caused by single microorganism type. In this study the most frequently isolated microorganism was E. coli with a rate of (75%). this result is agreement with findings of previous studies [28-33]. Uropathogenic Escherichia coli (UPEC), is one of the main causes of community (80–90%) and nosocomial acquired UTIs (30–50%) [34-37]. Also, our results reveal higher prevalence of urinary tract infections in female patients than in male [38-40]. this mainly due to short urethra, absence of prostatic secretion, pregnancy, and easy contamination of the urinary tract with fecal flora [41]. The emergence of high rates of antibiotic resistance and MDR-phenotype from urinary tract infections related bacteria becomes a public health concern worldwide. Our results findings that E. coli isolated were showed the following resistance rates; Cefpodoxime (100%), floxacin (90.8%), Ciprofloxacin (77.5%), Amikacin (60.8%) ceftriaxone (58.3%), Cefepime (51.7%), imipenem (11.7%), Colistin (0.8%). These results agreed with results of [42-46]. There is an increase in resistance of Uropathogens to most antibiotics because of excessive and inappropriate usage, reducing. These findings are clearly alarming as our country could be running out of effective antibiotics if this trend continues.

Current studies mainly focus on a variety of function enzymes produced in E. coli, including ESBLs, plasmid mediated AmpCs, and carbapenemases. Global emergence and spread of carbapenemase genes and ESBL genes among E. coli isolates, poses severe challenges to public health. Furthermore, this study indicated that ESBL positivity was closely related to the resistance of most drugs. In recent years, multidrug resistant caused by ESBLs are reported to be associated with higher morbidity and mortality rates [48]. The proportion of the ESBL positive cases was highest, followed by AmpC-producing stains, and carbapenemases-producing stains. ESBLs are mainly mediated by plasmid, while AmpCs are mainly mediated by chromosome. CTX-M types are the major phenotypes of domestic ESBLs, Widespread dissemination of these genes has been described in Africa and elsewhere, followed by SHV type [49]. AmpC genotype is given priority to blaCMY in the worldwide, especially the subtype of blaCMY-G2 [50]. In the present study, prevalence of β-lactamases producing isolates were found in (Table 4). Resistance to carbapenems can be acquired through mechanisms such as drug efflux, loss of porins, and carbapenemase-production [51], the latter of which is predominantly caused by the serine-carbapenemases such as K. pneumoniae carbapenemase (KPC) and oxacillinase -lactamase (OXA), or metallo- lactamases including Verona integron-encoded metallo- -lactamase (VIM), New Delhi metallo--lactamase(NDM), or imipenem as emetallo- -lactamase(IMP) [52]. New Delhi metallo-β-lactamase (NDM) and carbapenem-hydrolyzing oxacillinase-48 (OXA-48) are the most common carbapenemases among E. coli worldwide [53]. Metallo-lactamase-producing E. coli isolates were showed prevalence rates of 10.8%, 5.8%, 3.3 % and 2.5% for bla NDM, blaOXA-48, blaVIM and blaKPC, respectively; In this study, no blaIMP genes was found, but we cannot exclude that other MBL genes might have been involved in resistance.

Furthermore, reports of emerging (VIM, NDM, KPC and IPM,) MBL genes among Gram-negative strains have been published worldwide, including sub-Saharan Africa, Egypt [54], Uganda [55], Ethiopia [56], Kenya [57], South Africa [58], Saudi Arabia [59]. Thus, other carbapenemase-encoding genes should be evaluated in future studies and PCR should be conducted for detecting all carbapenemase-encoding genes in carbapenem resistant isolates. selecting antibiotics for treating bacterial infections should be according to the culture and sensitivity results and the international

guidelines to minimize further MDR development, cost-related and health-related consequences.

Conclusions

In this study, a high prevalence of resistance to β-lactams as well as to other antimicrobials were observed in E. coli isolates from DM patients with UTI. The study showed the importance of continuous monitoring programming of multidrug resistance in our hospitals. It also showed the need for developing attempts to decrease the prevalence of ESBL producing organisms and the modification of guidelines for UTIs. ESBLs are clinically significant and patients infected with ESBL-producing Enterobacteriaceae experience a greater likelihood of poor outcome if they are treated with inappropriate antibiotics. The exception is uncomplicated urinary tract infections where a very high urinary concentration of β-lactam antibiotics can be achieved.

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