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Prevalence of Beta-Lactamase Producing Genes in *Escherichia Coli* Isolated from Diabetic Foot Ulcers: A Hospital Based Study from South-Western Maharashtra

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Abstract

The antibiotic resistance in the bacteria inhabiting diabetic foot ulcers (DFU) is a major hurdle during medical treatment. Extended-spectrum beta-lactamase (ESBL) and Metallo-beta-lactamase (MBL) production in bacteria is one of the most prevalent beta lactam antibiotic resistance mechanisms. Resent literature suggested that *E. coli* is one of most abundant Gram negative bacteria found in DFUs. We studied the antibiotic resistance and the prevalence of the genes involved in beta lactamase production in the*E. coli* isolated from the DFU patients in Maharashtra, India. We isolated a total of 91 *E. coli* isolates from the diabetic patients and subjected to varies biochemical tests including ESBL and MBL tests. Antibiotic resistance in all the isolates was tested using Ampicillin, Amoxicillin, Piperacillin, Cefuroxime, Ceftriaxone, Cefoperazone, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicine, and Ciprofloxacin. The presence of TEM_{ESBL}, SHV_{ESBL}, $CTX\text{-}M_{ESBL}$,$NDM\text{-}1_{bla}$, KPC_{bla}, $OXA\text{-}48_{bla}$, and VIM_{bla} genes was evaluated by using specific primers and PCR. Among isolates 56.04% were positive for ESBL production and 48.35% were positive for MBL production. All the isolates showed varying degree of resistance towards antibiotics used. More than 50% isolates were vulnerable to carbapenem and aminoglycoside antibiotics. Genetic analysis revealed that $NDM\text{-}1_{bla}$ is the most prevalent gene is the *E. coli* isolates followed by TEM_{ESBL}, $CTX\text{-}M_{ESBL}$, and KPC_{bla}.VIM_{bla} is the least abundant gene found in the *E. coli* isolates. The results suggest that all *E. coli* isolates were multi-drug resistant. There was no association between the presence of a particular gene and antibiotic resistance in the isolates. Further studies considering all bacterial isolates from DFUs for antibacterial resistance and genetic characterization are necessary to understand the genetic basis of resistance.

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Key Words: Diabetic foot ulcer, E.coli, ESBL, MBL, NDM, TEM, VIM

Introduction

Diabetic patients and associated complications are increasing worldwide[1-2]. Diabetic foot ulcer (DFU) is one of the extreme medical conditions where patients may need to go through amputation of the infected part[3]. The bacterial infectionin DFU is one of the major hurdles during medical treatment. The bacterial infections in DFU are mono-cultural or involve multiple bacterial species which can be resistant to antibiotics. Though there are several antibiotic resistance mechanisms in bacteria inhabiting wounds have been described in literature [4-6], all of them are linked to the rich pool of genes, the resistome[7]. The genes involved in antibiotic resistance are widespread in producer organisms, pathogenic and non-pathogenic bacteria[5-6]. For example, CTX-M$_{ESBL}$ gene found in non-pathogenic bacteria is also prevalent in pathogenic bacteria [8-9]. Therefore, the genetic makeup of DFU wound inhabiting bacteria need to study as it could provide insight into the origin of antibiotic resistance mechanism.

Antibiotic resistant bacteria may have intrinsic or acquired genes that produce enzymes responsible for the deactivation or modification of antibiotics [5-6]. The production of beta lactamase enzymes responsible for the resistance to beta lactam antibiotics is one of the most common antibiotic resistance mechanisms found in the common pathogenic bacteria (e.g. *Escherichia coli* and *Klebsiella pneumonia*) in the family Enterobacteriaceae. Beta lactamase enzymes are further grouped into four categories based on the presence of Serine inthe active site and Zink-metalloenzymes. Beta lactamase enzymes such as Extended-spectrum beta-lactamases (ESBL) are derived from the genes TEM, SHV, and CTX-M [10]. In addition, Carbapenemase enzyme encoded by the genes (KPC, VIM, NDM, and OXA-48) located on the chromosomes or plasmid hydrolyses the beta lactam ring [11-12]. Moreover, many of the bacterial genes involved in beta lactam antibiotic resistance have cosmopolitan distribution [10-11]. However, studies on their prevalence in Indian population are limited [13].

India is a leading country with second largest population of diabetic patients. It is estimated that in India, nearly 25% diabetic patients will develop DFU [14-15]. Recently, Kale et al reviewed the etiology of the bacterial infection in DFU and reported that Gram negative bacteria including*E. Coli* are predominantly present in the DFU of the diabetic patients in India [16]. Several studies demonstrated that the pathogenic bacteria isolated from DFU are resistant to antibiotics [17-20]. However, the mechanism of antibiotic resistance in DFU inhabiting bacteria is rarely studied [19, 21-22]. Moreover, there are no studies describing the genetic basis of antibiotic resistance in the bacteria inhabiting DFU. The present study was undertaken to evaluate the antibiotic resistance in the*E. coli* bacteria isolated from DFU of patients, their mechanism of resistance (ESBL and MBL), and the prevalence of the genes responsible.

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Material And Methods

Sample collection

The study was carried out at Krishna Vishwa Vidyapeeth , Karad, Maharashtra, India. A total of 252 diabetic patients were screened for the bacterial infection in DFU. The data on age, sex, socio-economic status of the patients were recorded. Diabetic history and physiological conditions of each patient were recorded. Blood tests including sugar (glucose) level and HBA1C were performed before bacterial sampling. In addition, urea, creatinine, potassium, and sodium levels were determined following standard procedures.

Microbiological procedure

Pus samples were collected from the ulcer using two sterile cotton swabs. One swab was processed for gram staining and other for the culture on blood and MacConkey's agar. The inoculated plates were incubated at 37^{o}C. On next day, plates were observed for colony morphology and Gram staining. A total of 91 *E. coli* isolates were identified.

Biochemical characterisation

The tests for indol production, glucose and citrate utilization, and urea hybridization by *E. coli*isolates were confirmed following the standard protocols describedby CLSI. ESBl production was confirmed (reduced susceptibility to) by double disc synergy method using Ceftazidime and Calvulanic acid (30/10) mcg on Muller-Hinton agarfollowing CLSI guidelines. ESBL producer was determined based on ≥5 mm increase in the zone diameter of ceftazidime/clavulanic acid disc and ceftazidime disc alone and ≥5 mm increase in the zone diameter of cefotaxime/clavulanic acid disc and cefotaxime disc alone. Imipenem-EDTA disc method was used for detection of Metallo-beta-lactamase (MBL) producing isolates [23-24].

Antibiotic susceptibility testing

Antibiotic susceptibility of all *E. coli* isolates to Ampicillin, Amoxicillin, Piperacillin, Cefuroxime, Ceftriaxone, Cefoperazone, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicine, and Ciprofloxacin was studied by Kirby-Bauer disc diffusion following guidelines established by CLSI.

Genetic characterization of beta lactamase production

Genomic and plsmid DNA from the isolates were extractedby using the HipurA bacterial genomic DNA purification Kit and HipurA Plasmid DNA miniprep purification kit (HiMedia), respectivelyfollowingthe manufacturer's instructions. Betalactamase producing genes(TEM_{ESBL}, SHV_{ESBL}, CTX-M_{ESBL},NDM-1_{bla}, KPC_{bla}, OXA-48_{bla}, and VIM_{bla}) were amplified by Polymerase chain reaction using primers given in Table 1. PCR amplification was carried out in 20µL reaction mixture containing 1X PCR assay buffer (10 mMTrisHCl (pH 8.3), 1.5 mM MgCl2, 50 mM of KCl, 200 µM each dNTP, and 1U of Taq DNA polymerase; Merk Millipore), 0.2 nmole of each primer, and 200 ng of purified DNA template of each sample. The PCR reaction for amplification was carried out in a Master Cycler gradient PCR machine (Eppendorf). Detailed PCR programme is given in Table 2. Amplified products were analysed using 2.0% agarose gel electrophoresis in 1X TAE buffer. The gel was stained with ethidium bromide (10mg/ml), visualized under UV transilluminator, and photographed in gel documentation system (Bio- Rad Laboratories). *Klebsiella pneumoniae* ATCC 700603 was used as positive control strain for TEM_{ESBL}, SHV_{ESBL}, and

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CTX-M$_{ESBL}$. *Klebsiella pneumoniae* ATCC BAA-2146 and *K. pneumoniae* ATCC BAA-1705 strains ware used as positive controls for NDM-1$_{bla}$ and KPC$_{bla}$, respectively. The PCR product of OXA-48$_{bla}$ positive isolate (307 bp size) was confirmed by DNA sequencing and further used as positive control.

Results

The average age of the patients examined in the present study was 59.06 years (range 50-75 years). Supporting biochemical data of the patients is summarised in Table 3. A total 91 *E. coli* isolates were characterized biochemically and analysed for antibiotic resistance. All the isolates were positive for indole production, citrate and glucose utilization for energy, and hydrolysing urea for Nitrogen utilization.Among 91 isolates, 56.04% isolates were ESBL positive and 48.35% isolates were MBL positive.

MDR analysis revealed that more than 80% isolates were resistant to Ampicillin and Ciprofloxacin (Figure 3A). More than 50% isolates were vulnerable to Amikacin, Meropenem, Imipenem, Gentamicin, Cefoperazone, and Piperacillin (Figure 3A). The antibiotic resistance of the isolates obtained from male and female patients show similar trend (Figure 3B). The *E. coli* isolates obtained from female patients showed greater resistance to Cefepime (66.66% isolates in female vs 30% isolates in male) and Cefoperazone (50% isolates in female vs 38.18% isolates in males) as compared to the isolates obtained from male patients. Conversely, Piperacillin (54.54% isolates in male vs 38.88% isolates in females) and Meropenem (40% isolates in male vs 30.55% isolates in females) were more effective in inhibiting the growth of the isolates obtained from female patients than those from the male patients (Figure 3B).

PCR amplification revealed that NDM-1 was highly prevalent (61.53%) in *E. coli* isolates followed by TEM$_{ESBL}$ (51.64%), CTX-M$_{ESBL}$ (49.45), and KPC$_{bla}$ (46.15%; Table 4). The prevalence of these genes in the isolates obtained from male and female patientsdiffered from the general trend (Table 4). TEM was more prevalent in the isolates from male the patients (58.18%) while KPC$_{bla}$ (58.33%) was more prevalent in the isolates from the female patients (Table 4). The frequency of NDM-1$_{bla}$ was highest in the isolates irrespective of the presence of ESBL and MBL mechanisms of resistance. TEM was more prevalent in ESBL -ve (62.5%) and MBL +ve (59.09%) isolates (Table 4).The presence of a particular gene was not necessarily correlated with the antibiotic resistance (Figure 4). Most of the isolates wereresistant to Ampicillin and Ciprofloxacin irrespective of the presence of particular gene (Figure 4). Irrespective of the presence of a gene, a significant proportion of *E. coli* isolates were vulnerable to carbapenem (Cefepime and Meropenem) and aminoglycoside (Amikacin and Gentamicine) antibiotics (Figure 4).

Discussion

To develop effective strategies to control the infections in DFUs, the antibiotic resistance in inhabitant bacteria and their genetic basis need to be evaluated [25-26]. In this order we attempted to understand the antibiotic resistance in *E. coli* and the results revealed that all the isolates from DFUs, up to some extant showed resistance towards different antibiotics.

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Previously, multidrug resistance in DFU inhabiting bacteria including *E. coli* have been reported in several studies[13-20, 27-28]. In the present study, more than 80% isolates showed resistance to Penicillin and Ciprofloxacin while more than 50% isolates were vulnerable to carbapenems (Imipenem and Meropenem) and aminoglycoside (Amikacin and Gentamicin). Similar to these results [13], found that carbapenem antibiotics are effective against Gram negative bacteria including *E. coli*.

Beta lactam antibiotic resistance in pathogenic bacteria is one of the most prevalent antibiotic resistance mechanism [29] linked with the presence of beta lactamase producing genes [10-12]. We analysed the frequency of the genes involved in beta lactamase antibiotic resistance in one of the most common pathogenic bacteria *E. coli* and found that NDM-1 is the most prevalent gene in *E. coli* isolates followed by TEM, CTX-M, and KPC. The prevalence of the genes involved in ESBL and MBL resistance have been studied worldwide and their frequency in pathogens may depend on the geographical location [10-11]. For example, NDM-1 and its variants are disseminated in the Indian sub-continent [11, 30]. A few studies reported the presence of beta lactamase genes in the bacteria inhabiting DFU of the Indian population suggesting that they are widespread in different bacterial species[19, 31]. In the present study, for the first time we reported the prevalence of beta lactamase genes in *E. coli* isolates from DFU. Several previous studies on the genetic characterization of the pathogenic bacteria isolated from the medical samples other than DFUs reported the prevalence of the genes included in the present study [21-22, 32-36]. Among the genes involved in ESBL production, TEM and CTX-M were present in almost 50% of *E. coli* isolates. Conversely [22], reported the lowest prevalence of TEM (9.09%) and CTX-M (3.03%) genes in *E. coli* isolates while the studies by [33-36] reported greater prevalence of both the genes. We observed the presence of SHV gene in 27.47% *E. coli* isolates. Previously, Khajuria [36] reported the presence of SHV gene in 62% isolates and Gupta et al [34] reported in 40.48% isolates. Further, NDM-1 gene reported to be prevalent in more than 60% *E. coli* isolates [33-34, 37-38]. However, Rahman et al [36] reported the presence of NDM-1 gene in 45.5% *E. coli* isolates. We observed the lowest proportion of VIM (12.08%) and OXA-48 (13.18%) genes in the present study. Similarly, Govindaswamy et al [33] and Nagraj et al [37] reported the low prevalence of VIM gene while Gupta et al [34] could not detect VIM in *E. coli* isolates. The prevalence of OXA-48 varies (5.3%-42%) in the *E. coli* isolates reported in different studies [33-36]. In the present study, we detected KPC gene in 46.15% isolates. Previous studies reported the presence of KPC gene in very low proportion [33] (Govindaswamy et al., 2019) in *E. coli* isolates while Gupta et al [34] reported the absence of KPC gene in the *E. coli* isolates from symptomatic patients. Combined these results suggests that the prevalence of the beta lactamase producing genes in the *E. coli* bacteria from the Indian patients varies in different pathological conditions. Further studies on the genetic characterization of the beta lactamase producing bacteria in different pathological conditions need to be undertaken to have baseline information about the antibiotic resistance in Indian population.

DFUs may have mono-microbial or poly-microbial infections [16]. In the poly-microbial infections, bacteria cooperate and establish symbiotic relations which confer the maintenance of the infection and sometimes the production of toxins [3]. In poly-microbial infection, each

species of bacteria may have different gene pool and able to secrete variety of enzymes which could contribute to the antibiotic resistance and delayed wound healing [3, 7, 40]. Beta lactamases can be found in the outer membrane vesicles or extracellular vesicles of the bacterial cell wall and extra cellular spaces around producers. These beta lactamases can protect producers and entire poly-microbial colony as well from antibiotics [41]. Moreover, the biofilm formation by pathogenic bacteria in diabetic foot infections is also an emerging issue having contribution to the antibiotic resistance [42-43]. The bacteria inhabiting poly-microbial colony are also able to exchange genes through horizontal transfer and produce chemicals that alter antibiotic tolerance, drug sensitivity, and cell wall [6, 41]. Therefore, to understand the complete scenario, antibiotic resistance in different bacterial isolates from DFUs and their combined gene pool need to be evaluated.

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Figure 1 Representative agarose gel images of TEM (A), SHV (B), and CTX-M (C) Genes. 100 bp DNA ladder was loaded in the first well; -ve and +ve controls were loaded in lane 2 and lane 3, respectively. Remaining five wells were loaded with the PCR product of the genes amplified using the DNA of clinical isolates. Blank lanes indicated no amplification.

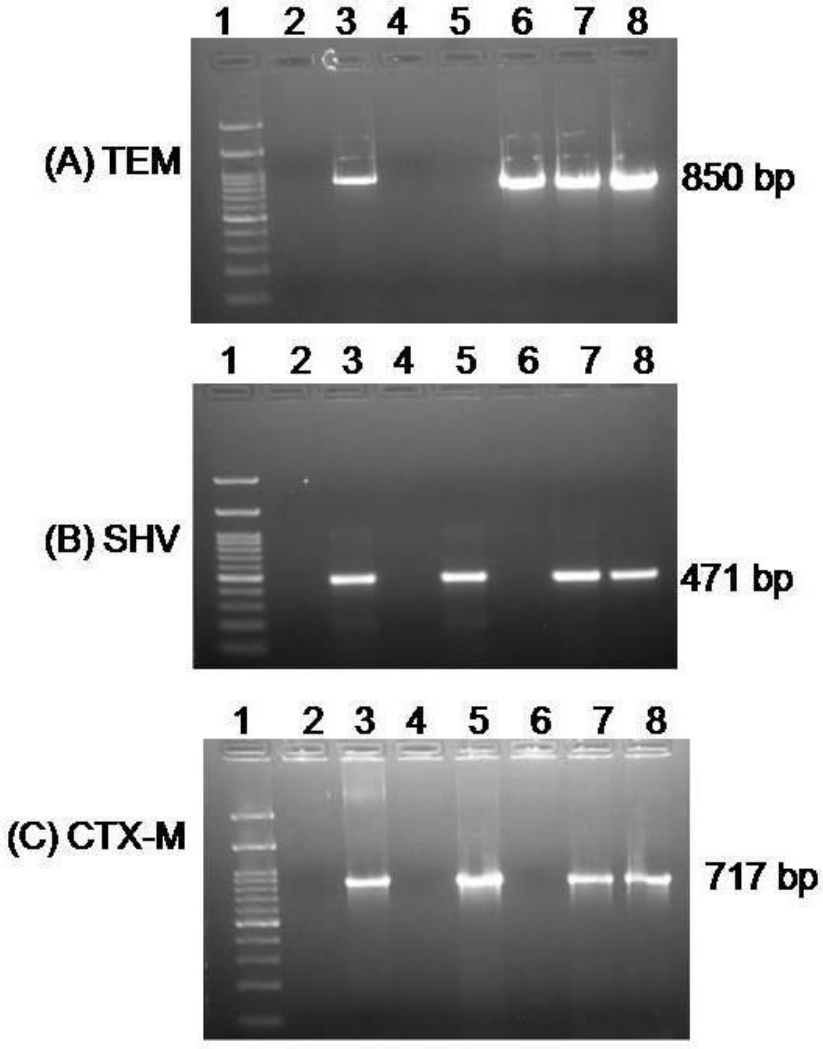

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Figure 2 Representative agarose gel images of NDM (A), KPC (B), OXA-48 (C), and VIM (D) Genes. 100 bp DNA ladder was loaded in the first well; -ve and +ve controls were loaded in lane 2 and lane 3, respectively. Remaining five wells were loaded with the PCR product of the genes amplified using the DNA of clinical isolates. Blank lanes indicated no amplification.

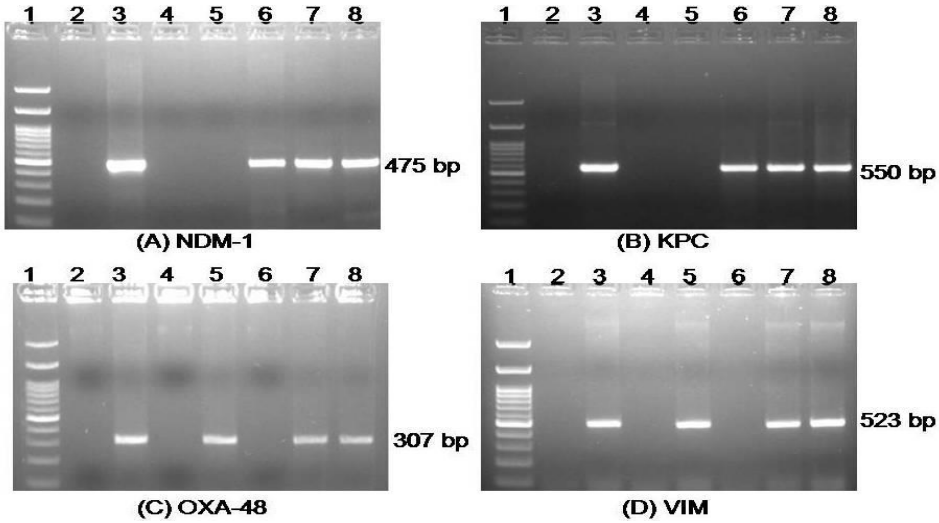

Figure 3 Proportion of antibiotic resistant isolates. (A) Overall proportion of isolates resistant to different antibiotics. (B) Proportion of antibiotic resistant isolates obtained from male and female diabetic patients.

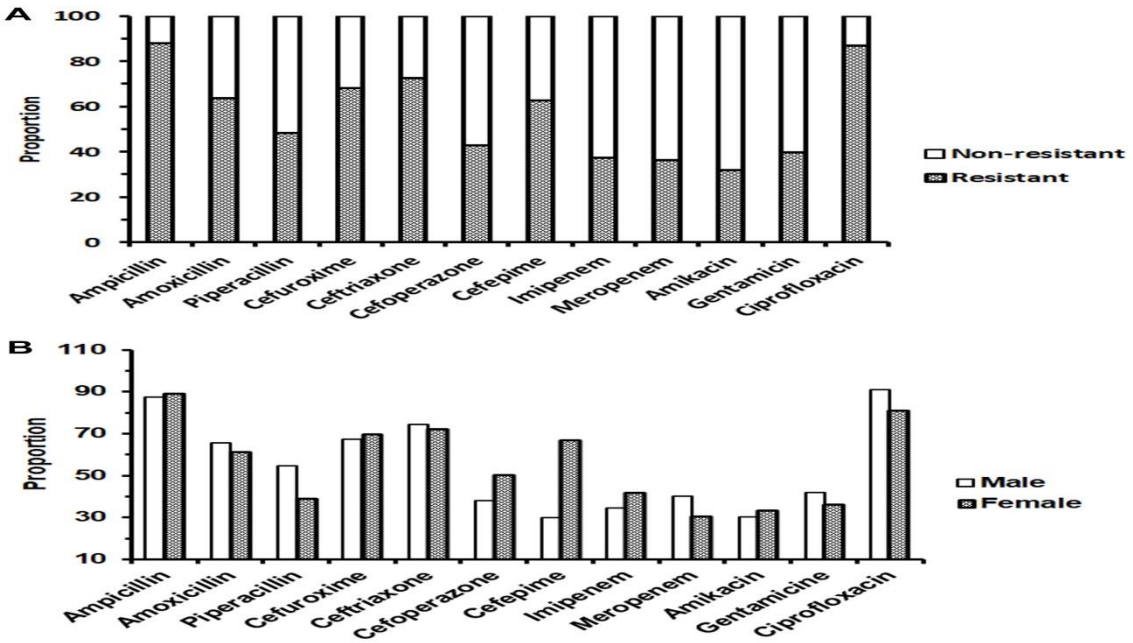

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Figure 4 Heat map depicting the proportion of antibiotic resistant *E. coli* isolates with different genes involved in antibiotic resistance.

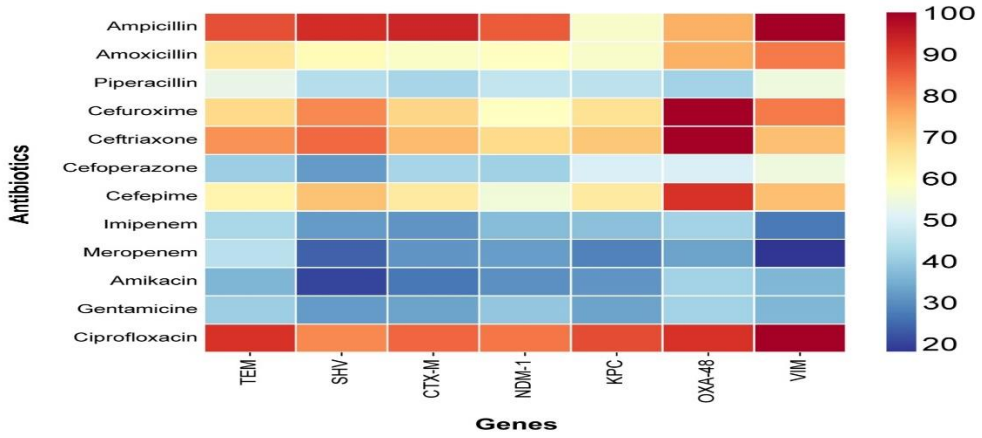

Table 1 Details of the primers used in the present study for the amplification of the genes involved in ESBL (TEM, SHV, and CTX-M) and MBL (NDM-1, KPC, OXA-48, and VIM) resistance.

Primer name	Primer sequence	Amplicon size
TEM (bla TEM)	F 5'- ATG AGT ATT CAA CAT TTC CG-3'	850 bp
	R 5'- CCA ATG CTT AAT CAG TGA GG-3'	
SHV (bla SHV)	F 5'-CAG CGA AAA ACA CCT TG-3'	471 bp
	R 5'-CCG CAG ATA AAT CAC C-3'	
CTX-M (bla CTX-M)	F 5'-GGT TAA AAA ATC ACT GCG TC-3'	717 bp
	R 5'-TTG GTG ACG ATT TTA GCC GC-3'	
NDM-1	F 5'-GGGCAGTCGCTTCCAACGGT-3'	475 bp
	R 5'-GTAGTGCTCAGTGTCGGCAT -3'	
KPC	F 5'-GCT CAG GCG CAA CTG TAA G-3'	550 bp
	R 5'-AGC ACA GCG GCA GCA AGA AAG-3'	
OXA-48	F 5'-GCGTGGTTAAGGATGAACAC-3'	307 bp
	R 5'-CGCTCCGATACGTGTAACTT-3'	
VIM	F 5'ATTGCCGATGGTGTTTGG-3'	523 bp
	R 5'TGGGCCATTCAGCCAGA-3'	

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Table 2 Detailed programme of polymerase chain reaction for the genes included in the present study.

		TEM (*bla TEM*)	SHV (*bla SHV*)	CTX-M (*bla CTX-M*)	NDM-1	KPC	OXA-48	VIM
Initial denaturation	Initial denaturation	95°C-5 min	95°C-5 min	95°C-5 min	95°C-10 min	95°C-10 min	95°C-10 min	95°C-10 min
		1 cycle	1 cycle	1 cycle	1 cycle	1 cycle	1 cycle	1 cycle
Amplification	Denaturation	95°C-20 sec	95°C-20 sec	95°C-20 sec	95°C-30 sec	95°C-30 sec	95°C-30 sec	95°C-30 sec
		30 cycle	30 cycle	30 cycle	30 cycle	30 cycle	30 cycle	35 cycle
	Annealing	55°C-20 sec	45°C-20 sec	52°C-20 sec	55°C-30 sec	55°C-30 sec	55°C-30 sec	45°C-30 sec
		30 cycle	30 cycle	30 cycle	30 cycle	30 cycle	30 cycle	35 cycle
	Extension	72°C-20 sec	72°C-20 sec	72°C-20 sec	72°C-30 sec	72°C-30 sec	72°C-30 sec	72°C-30 sec
		30 cycle	30 cycle	30 cycle	30 cycle	30 cycle	30 cycle	35 cycle
Final extension	Extension	72°C-5 min	72°C-5 min	72°C-5 min	72°C-10 min	72°C-10 min	72°C-10 min	72°C-10 min
		1 cycle	1 cycle	1 cycle	1 cycle	1 cycle	1 cycle	1 cycle

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Table 3 Biochemical test results of 252 patients included in the present study. Values are expressed as average of all patients and range of the values in given in parenthesis.

Test	Values
HBA1C	7.4 (6.1-9.6) mmol/mol
Blood Sugar	225.09 (152-350) mg/dL
Urea	27.79 (18.3-45) mg/dL
Creatinine	0.96 (0.6-1.8) mg/dL
Sodium	140.54 (136-148) mEq/L
Potassium	4.10 (3.8-5.1) mEq/L

Table 4 Presence of various genes in *E. coli* isolates with respect to sex of the patients and resistance mechanism. Values are presented in percentile and the numbers in parenthesis are actual numbers of the *E. coli* isolates (ESBL: Extendedspectrum beta-lactamase; MBL: Metallo-beta-lactamase).

	Total (91 patients)	Male (55)	Female (36)	ESBL +ve (51)	ESBL -ve (40)	MBL +ve (44)	MBL -ve (47)
TEM	51.64 (47)	58.18 (32)	41.66 (15)	43.13 (22)	62.5 (25)	59.09 (26)	44.68 (21)
SHV	27.47 (25)	30.09 (17)	22.22 (8)	31.37 (16)	22.5 (9)	22.72 (10)	31.91 (15)
CTX-M	49.45 (45)	43.63 (24)	58.33 (21)	45.09 (23)	55 (22)	52.27 (23)	46.8 (22)
NDM-1	61.53 (56)	56.36 (31)	69.44 (25)	60.78 (31)	62.05 (25)	63.63 (28)	59.57 (28)
KPC	46.15 (42)	38.18 (21)	58.33 (21)	49.01 (25)	42.05 (17)	45.45 (20)	46.8 (22)
OXA-48	13.18 (12)	9.09 (5)	19.44 (7)	9.8 (5)	17.5 (7)	15.9 (7)	10.63 (5)
VIM	12.08 (11)	9.09 (5)	16.66 (6)	17.64 (9)	5 (2)	6.81 (3)	17.02 (8)