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Molecular Characterization of Resistance to ESBL Producing *E.coli* and Development of LAMP Assay against the Microbes for UTIs Patients

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ABSTRACT

Most of the Urinary Tract Infections (UTIs) are caused by multidrug-resistant bacteria mostly E. coli which produce Extended Spectrum β-Lactamase (ESBL). The prevalence of UTI infection caused by ESBL producing bacteria is much worldwide and associated with rising health care costs. To identify the pattern of multidrug resistance amongst the E. coli isolates which were obtained from the patients of UTI. We found antibiotic resistance rates as: ampicillin (100%), amoxicillin (100%), cefotaxime (85.7%), tetracycline (88.5%), amikacin (57.1%), nitrofurantoin (65.7%), gentamicin (71.4%), ciprofloxacin (82.8%), chloramphenicol (94.2%), norfloxacin (77.1%), ceftazidime (62.8) and amoxicillin/clavulanic acid (0%). ESBL production was found to be 34.28% by using clavulanic acid as a β-lactamase inhibitor in the phenotypic. The ESBL-encoding genes were characterized for CTX-M, TEM, and SHV genes, CTX-M was found to be 85.4%, TEM 12.8% and SHV 6.5% in isolates of E. coli. Apart from this, we also developed a highly sensitive and specific LAMP assay for ESBL producing E. coli. The assay is rapid (results can be obtained in less than 1 hour), cost-effective easy to perform, and it will be very easy to adapt in small-scale industries, hospitals, and testing laboratories.

Keywords: Diabetes mellitus, Urinary tract infections (UTIs), Extended-spectrum β-lactamase (ESBL), Drug-resistant, *E. coli*, Loop-mediated isothermal amplification (LAMP)

INTRODUCTION

According to the National Institutes of Health (NIH), the second most common infection affecting women, the elderly, and infants are the urinary tract infections (UTIs). Acute UTI is one of the common acute bacterial infections, mostly gram-negative bacilli. Approximately 80% of UTIs is caused by *E. coli* in both the acquired community and the hospitalized patients [1]. An estimated 150 million cases of community-acquired UTIs are diagnosed worldwide every year and the increase in resistance towards β-lactamases antibiotics [2-5]. More than 700 different types of β-lactamases have been illustrated and can be divided into 3 major groups of ESBL enzymes; TEM, SHV, and CTX-M further can be divided into some subgroups (http://www.lahey.org/studies) [6]. Among them, CTX-M reported more than 100 types of enzyme, categorized on the basis of amino acid sequences. CTX-M variants have been divided into 5 subgroups, CTX-M1, CTX-M2, CTX-M8, CTX-M9 and CTX-M25 [7,8]. During the last decade, CTX-M enzymes have become the most prevalent ESBL enzymes, especially in ESBL producing *E. coli* in Europe, Asia and South America [9,10]. The ESBL producing bacterium may contain genes for one or more of these enzymes that are coded by several types of *bla* (β-lactamase) genes, based on structural similarity [11]. These *bla* genes most often carry resistance determinants for broad-spectrum cephalosporins and making the microorganisms resistant to third-generation cephalosporin, broad-spectrum antibacterial drugs [12-14].

We have developed Loop-mediated isothermal amplification (LAMP) assay for detection of *bla* genes in *E. coli*. The

LAMP is a rapid and specific technique for DNA amplification [15]. It requires 4 primers targeting 6 regions within a gene. Therefore, the present study uses methods of molecular typing for ESBL positive *E. coli* isolates by using PCR which has been developed for the identification of the β-lactamases *bla* genes and LAMP assay against the microbes for UTIs patients.

PATIENTS AND METHODS

Patients and Sample Collection

The urine samples from 100 different patients were collected in sterile plastic universal containers and were transported without delay under ice-cold conditions by adding boric acid at a final bacteriostatic concentration of 1.8% [16]. Out of 100 samples, 35% had the presence of *E. coli* (Table 1). The isolates were initially cultured from received urine samples. For the culture microbes, a loop full of the infected urine sample was streaked on the CLED (Cystine lactose electrolyte deficient medium) and Mac-Conkey agar medium and immediately incubated at 37°C for 24 hours [17]. The plates were examined after 24 hours of incubation. For tentative identification of *E. coli*, colonies were selected and characterized on the basis of Gram's staining, their morphology, cultural and biochemical characteristics were characterized according to the key of Bergey's Manual of Systematic Bacteriology [18,19]. The cultured samples were stored in cryovials containing Luria-Bertani broth with 50% glycerol (Invitrogen, USA) at -20°C. All the experiments were done in triplicates and the results were calculated as mean along with one standard deviation (SD) [20].

Table 1 Details of microbes in the collected urine sample during the study period

S. No.	Microbes	No. of positive cases	Percentage (%)
1	*Escherichia coli*	35	35%
2	*Klebsiella pneumoniae*	20	20%
3	*Pseudomonas aeruginos*	15	15%
4	*Proteus vulgaris*	15	15%
5	*Staphylococcus aureus*	10	10%
6	*Proteus mirabilis*	5	5%

Antimicrobial Susceptibility Testing and Screening for ESBL

Antimicrobial susceptibility testing was performed for all *E. coli* isolates by using the Mueller-Hinton agar disc diffusion method [21]. A panel of antimicrobial agents, ampicillin, amoxicillin, cefotaxime, tetracycline, amikacin, nitrofurantoin, gentamicin, ciprofloxacin, chloramphenicol, norfloxacin, ceftazidime and clavulanic acid (Sigma Aldrich, United State) were tested and MICs were recorded using the Sensititre Vizion System (Trek Diagnostic Systems) (Table 2). The phenotypic confirmation of ESBL production was confirmed by using disc diffusion with discs of cefotaxime and ceftazidime/clavulanic acid, ceftazidime, and ceftazidime/clavulanic acid [22,23]. The inhibition zone of the cephalosporin plus-clavulanate disc was equal to or a greater than 5 mm increase in diameter, when compared to the cephalosporin only and interpreted as phenotypic evidence of ESBL production. The double-disk synergy test was performed according to established protocols and the results were calculated as mean with standard deviation of one SD [20].

Table 2 List of antimicrobial drug used with quantity

S. No.	Drug	Quantity (µg)
1	Ampicillin	10
2	Amoxicillin	20
3	Cefotaxime	30
4	Tetracycline	30
5	Amikacin	30
6	Nitrofurantoin	300
7	Gentamicin	30
8	Ciprofloxacin	5
9	Chloramphenicol	30
10	Norfloxacin	10
11	Ceftazidime	30
12	Clavulanic acid	30

DNA Extraction and Amplification by PCR

Template DNA for Polymerase Chain Reaction (PCR) was extracted from freshly cultured bacteria by using a DNeasy (Qiagen, Germany) according to the instructions of the manufacturer. Isolated DNA was used for screening for SHV, TEM, and CTX-M genes, the primers sequences for all 3 genes are respectively, SHV (F): ATG CGT TAT ATT CGC CTG TG, SHV (R): TGC TTT GTT CGG GCC AA [24]; TEM (F): GCC AAC TTA CTT CTG ACA AC, TEM (R): GCC AAC TTA CTT CTG ACA AC [25]; and CTX-M group CTX-M (F): CGC TTT GCG ATG TGC AG , CTX-M (R): ACC GCG ATA TCG TTG GT [26]. PCR reactions were carried out in 50 µl reaction tubes using specific primers and annealing temperature for amplifying the TEM, SHV, and CTX-M genes. Total volume of 25 µl containing; 2.5 µl of 10X PCR reaction buffer, with $MgCl_2$ (1.5 mM), 0.5 µl of dNTPs (10 mM), 0.5 µl of each primer (10 pm/µl), 0.5 µl (2 U/µl) Taq DNA polymerase (Sigma Aldrich, United State) and 50 ng DNA template. Amplifications were performed in a thermal cycler (Eppendorf, USA), using the following conditions: 95°C for 2 min; 35 cycles at 94°C for 30 s, 55°C for 50 s, and 72°C for 50 s; final extension at 72°C for 2 min. PCR amplified product was analyzed in 2% agarose gel electrophoresis at 10 V/cm that was stained with ethidium bromide, visualized under ultraviolet (UV) light and photographed using gel documentation system (Image Master VDS, Pharmacia, USA).

LAMP Assay

Four specific oligonucleotide primers, two outer primers (F3 and B3) as used in regular PCR and two inner primers, forward inner primer (FIP), back inner primer (BIP) were used for the *E. coli* LAMP assay. All primer sequences were designed for analyzing a conserved region of the *E. coli* β-lactamases genes with the software program Primer Explorer V 3 (http://primerexplorer.jp/elamp3.0.0/index.html). The primers were selected based on the criteria described by Notomi, et al [27]. The design of the two outer primers, F3 and B3, is the same as that of regular PCR primers, while the design of the two inner primers, FIP and BIP, is different from that of PCR [27]. FIP consists of the sense sequence of F2 at the 3′ end and the F1c region at the 5′ end that is complementary to the F1 region. BIP consists of a B2 region at the 3′ end that is complementary to the B2c region and the same sequence as the B1c region at the 5′ end. The LAMP reaction relies mainly on auto-cycling strand displacement DNA synthesis that is similar to the cascade rolling-circle amplification reported by Hafner, et al [28]. Two pairs of primers, including F3-GCA ATG GCG CAA ACT CTG, B3- TTG GTG GTG CCA TAG TCA C, FIP- TCA TCC ATG TCA CCA GCT GCG-AAT CTG ACG CTG GGT AAA GC and BIP- AGG CAA TAC CAC CGG TGC AGC-GGT TTT ATC CCC CAC AAC C were used in LAMP amplification. The LAMP reaction mixtures (25 µl) contained one pair of outer primers (0.2 µM) and one pair of inner primers (1.6 µM), 2.5µl of 10 × Bst DNA polymerase reaction buffer [1µl containing 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 (pH 8.8), 1µl of an 8U µl/1 concentration of Bst DNA polymerase (New England Biolabs, MA), 400 µM each dNTP, 2 mM $MgSO_4$ (2 µl), 5 µl of betaine (Sigma Aldrich, United State), and 5 µl of DNA sample same as used for PCR. The LAMP reaction was performed in a heating block at 65°C for 1 hour [15]. For comparison, the reaction was also performed at the same conditions by using a conventional thermal cycler (Eppendorf, USA). Visualization of the LAMP products was performed through observation of a color change following the addition of 1 µl (1:1,000) of SYBR Green I® (Sigma-Aldrich, United States) in the reaction of the tube. This changed color was visualized by the naked eye and produced fluorescence in under UV source (UV transilluminator).

RESULTS

In the present study, a total of 100 UTI isolates were collected for the culture. These isolates were identified using conventional cultural, biochemical and morphological tests (*P. aeruginosa*, *P. mirabilis*, *K. pneumoniae*, *E. coli*, *P. vulgaris,* and *S. aureus*), the results were summarized in Table 1. Antibiotic sensitivity test was carried out in all the 35 *E. coli* isolates and prevalence of resistance were ampicillin (100%), amoxicillin (100%), cefotaxime (85.7%), tetracycline (88.5%), amikacin (57.1%), nitrofurantoin (65.7%), gentamicin (71.4%), ciprofloxacin (82.8%), chloramphenicol (94.2%), norfloxacin (77.1%), ceftazidime (62.8) and amoxicillin/ clavulanic acid (0%) (Table 1 and Figure 1). These isolates were also identified for ESBL production by using clavulanic acid as a β-lactamase inhibitor in the phenotypic confirmatory disk diffusion test and found 35% (35/100) isolates to be ESBL producers. ESBL-

producing isolates to possess a higher degree of resistance towards antibiotics as compared to non ESBL producing isolates.

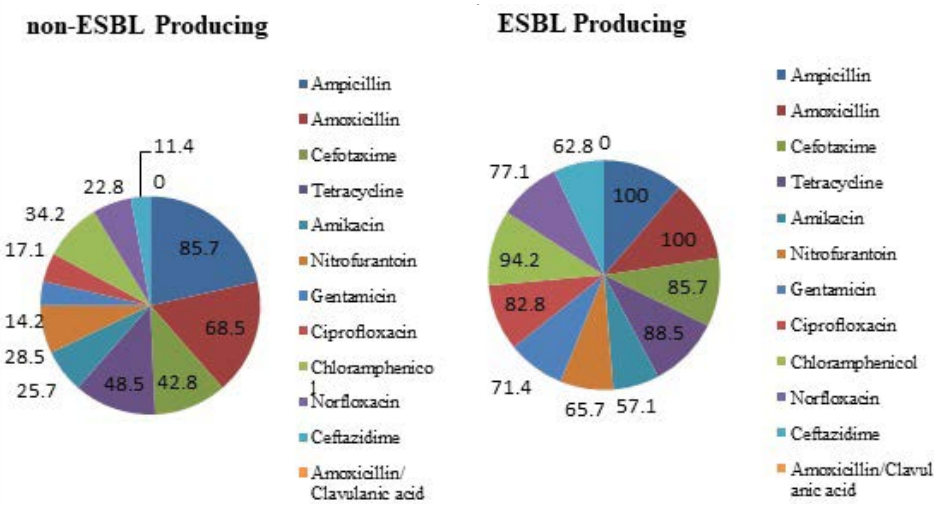

Figure 1 Antibiotic resistance and suitability rates in non-ESBL and ESBL producing *E. coli* isolated in this study

The ESBL encoding genes of all 12 isolates were characterized by PCR on overall phenotypic results obtained with ESBL tests. The specific primers were used on the isolated DNA of ESBL producing isolates for the characterization of TEM, SHV, and CTX-M genes. Among these isolates, a majority of CTX-M was found in 8 (85.4%) isolates of *E. coli*. SHV was found in 3 (6.5%) isolates of *E. coli*. TEM was detected in 5 (12.8%) isolates of *E. coli*. The positive PCR products of the CTX-M, TEM, and SHV were obtained in different sizes respectively.

The LAMP assay was designed for the detection of ESBL producing *E. coli*. All 12 isolates were detected by LAMP assay. Primers were specifically designed, two outer primers (F3 and B3), two inner primers (FIP and BIP) used in the amplification. Each inner primer contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA and form stem-loop structures at both ends of the minimum LAMP reaction unit. The assay showed 100% specificity for ESBL producing *E. coli* with no false-positive or false-negative results. This indicates the high specificity of the LAMP assay for targeting isolates detection. The sensitivity of LAMP assay was tested by 10-fold serial dilutions of isolated DNA. The assay showed sensitivity to target as little as 1 pg of isolated DNA, whereas PCR as little as 10 pg of isolated DNA. The detection of LAMP product is visualized by naked eyes after addition of SYBR green I dye to each reaction tube. The color change showed the positive result of observing the amplification products; otherwise, it remained unchanged (Figure 2). The LAMP products were also visualized by placing the reaction tube on a UV transilluminator at 302 nm (Figure 3). All isolates showed positive reaction within 30 minutes.

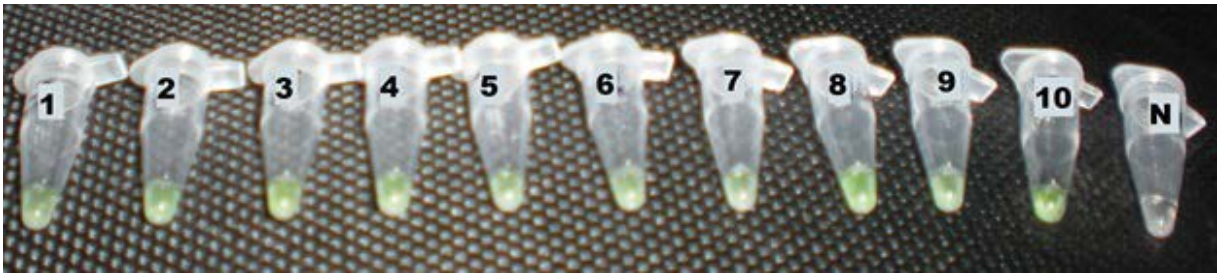

Figure 2 The LAMP results after adding SYBR Green dye

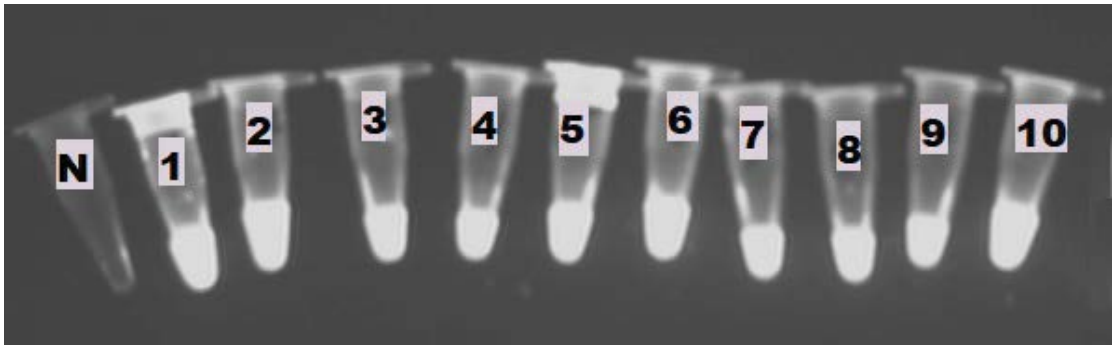

Figure 3 The LAMP results visualized by placing the reaction tube on a UV transilluminator

DISCUSSION

Over the last few years, a significant increase in the number of ESBL-associated *E. coli* has being reported in several parts of the world [29,30]. A frequent irrational use of antibiotics changes the intestinal flora, leading to bacterial resistance [31]. In this study, we focused on the UTI *E. coli* isolates and their sensitivity patterns to different groups of antibiotics which were commonly administered to treat the UTI. Our results showed that 34.28 % (12/25) isolates were found to be produced by ESBL. A prior study reported a similar finding, the prevalence of the ESBL producers (39.8%) in burn patients [32]. The incidence of resistance was found 76.5% multidrug-resistant to more than two antibiotics in non ESBL producing isolates and 100% resistance to some antibiotics in ESBL producing isolates. As ampicillin and amoxicillin showed 100% resistance in ESBL producing isolates but it was 65% and 43% respectively in non-ESBL producing isolates. The incidence of ESBL-producing strains has been reported to be 68% (69 of 101) in China and from the United States, it was found to be 40% (60 of 150) [33,34]. Harakuni, et al., reported a high prevalence of the ESBLs (74%) in ICU patients [35]. A high incidence of multidrug-resistant isolates was also detected in the present isolates. It has been proved that the prevalence of the ESBLs among the clinical isolates varies from country to country and institution to institution within the same country, due to clinical failure of empirical treatment protocols [36]. The presence of ESBL has been associated with increased mortality, longer duration of hospitalization and increased hospital costs [37-41].

Molecular detection and identification of β-lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. Genotyping of ESBL encoding genes indicated that β-lactamase families associated with high spectrum activity against β-lactamases and found 3 types of ESBL genes in this study: CTX-M, SHV, and TEM. CTM-X genes have a high occurrence than TEM and SHV genes; CTX-M was found to be 85.4%, TEM (12.8%) and SHV (6.5%) respectively. These findings are in accordance with previous literature where CTX-M genes were found to be quite high in clinical isolates in a study conducted in France and Chinese urban river [42,43]. Most of the ESBL producers contained the ESBL type CTX-M gene [44-46].

In this study, rapidity, sensitivity, specificity, and robustness of the LAMP assay were established. LAMP assay was found to be highly sensitive and it detected as little as 1 pg of isolated DNA of *E. coli*. The high sensitivity comes from its ability to detect a few copies of DNA in the reaction mixture [47]. LAMP positive reactions were detected by visual color change after adding SYBR Green. In conclusion, the rapid, specific, sensitive, and simple platform of LAMP assays may present another valuable tool for detection of ESBL producing *E. coli.* Due to the shorter reaction time and better visual results of positivity without requiring sophisticated instruments, the LAMP assay can be more easily applied in the field of laboratories than PCR [15,47]. However, the disadvantages of PCR assay are more instruments requirement and time consumable than the LAMP method [48]. LAMP amplification is rapid easy to perform, and low in cost, it will be very easily adapted in small-scale industries, hospitals, and testing laboratories.

CONCLUSION

In this study, we found *E. coli* was more frequent microbes in UTI patients and the spread of drug resistance among them. ESBL producing *E. coli* was a most antibiotic-resistant pathogen in UTI. Genotyping of ESBL producing genes indicated that β-lactamase families associated with high spectrum activity against β-lactamases and found 3 more common types of ESBL genes: CTX-M, SHV, and TEM. In Addition, this study provides an easy, fast and simple

LAMP based diagnostic tool for ESBL producing *E. coli.* LAMP based detection could contribute to cheap and easy diagnostic tool in small-scale industries, hospitals, and testing laboratories.

DECLARATIONS

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Conflict of Interest

The authors have disclosed no conflict of interest, financial or otherwise.

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