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Original Article

Antibiotic Sensitivity, ESBL production and Prevalence of *bla*$_{SHV}$ and *bla*$_{OXA}$ genes in *Escherichia coli* from Urinary Tract Infection

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Ninety five *Escherichia coli* isolates from diagnosed urinary tract infections were examined for antibiotic resistance, ESBL production and the presence of two ESBL producing genes *bla*$_{SHV}$ and *bla*$_{OXA}$. It was observed that the prevalence of UTI was most in ages between 19 to 45 years. All the isolates were resistant against different antibiotics including the third generation cephalosporins. Resistance against ciprofloxacin, the frequently prescribed drug was 82%. Only meropenem and nitrofurantoin showed greater sensitivity towards the isolates showing 13 % and 33 % resistance respectively. The resistances were 90, 86, 82 , 79 , 78, 76, 75, 75, 74, 73, 72, 68, 33 and for amoxicillin, nalidixic acid, ciprofloxacin, cephalexin, ceftriaxone, cefixime, amoxiclav, ceftazidime, netilmicin, aztreonam, gentamicin, amikacin, nitrofurantoin and meropenem respectively. Thirtyone isolates were randomly selected for detection of ESBL Production by double disc diffusion synergy test. Twenty one isolates (67.74%) were positive in ESBL production. PCR experiments were carried out using the 21 ESBL positive *E. coli* isolates to examine the presence of two ESBL genes namely *bla*$_{SHV}$ and *bla*$_{OXA}$. All (100%) of the 21 isolates showed the presence of *bla*$_{OXA}$ gene, whereas 8 (38%) isolates among the 21 showed the presence of *bla*$_{SHV}$ gene.

Key words: ESBL, *bla*$_{SHV,}$ *bla*$_{OXA}$, urinary tract infection (UTI)

Introduction

During the recent years, extended spectrum beta lactamase producing pathogens are being detected quite frequently in Bangladesh. The present investigation studied the occurrence of multi drug resistance among *E. coli* isolates from urinary tract infection, the occurrence of ESBL producing *E. coli* and detection of two common ESBL genes, namely *bla*$_{SHV}$ and *bla*$_{OXA}$. ESBL, and ESBL-like, genes can be found both chromosomally or on transferable genetic elements such as plasmids. They are predominantly found in Enterobacteriaceae. SHV and OXA can be found both on chromosomal and plasmid DNA although these are predominantly found on plasmids[1]. Therefore, this study used both genomic and plasmid DNA as a template for the isolation of these genes.

The present study focused on clinical isolates from urinary tract infections. The samples were randomly taken regardless of age, sex and socioeconomic status of the patients. It is estimated that there are about 150 million urinary tract infections per annum worldwide[2]. It also includes the most common nosocomial infection in many hospitals and accounts for approximately 35% of all hospital acquired infections. The most common etiological agent in UTI is *E. coli* from uncomplicated urinary tract infection isolates[3-4]. It was first observed in 1983 in isolates of *Klebsiella pneumoniae*[5]. The extended spectrum β-lactamase (ESBL) producing strains have variable susceptibility rates for fluoroquinolones, aminoglycosides, and fourth-generation cephalosporins[3,6] The incidence of extended spectrum β-lactamase producing enterobacteriacae has increased worldwide since their first description about two decades ago[7-8]. A recent survey in the US estimated that, 6 % of all *Escherichia coli* and 12% of all *Klebsiella* spp. isolates produce ESBL[9-10]. The European rates are higher, but show a variation between 2.7% and 30% for *E. coli*[10]. In 2008, the Sentinel Surveillance of Antibiotic Resistance in Switzerland found third generation cephalosporin resistance among 4.2% of tested *E. coli*. Infections by ESBL-strains cause increased morbidity and mortality warranting adequate ESBL surveillance[11]. Early detection of ESBL-producing pathogens is considered important in order to implement efficient infection control measures and to select appropriate antimicrobial treatment regimens[12].

Most ESBLs are derivatives of TEM or SHV enzymes[13-14]. TEM and SHV-type ESBLs are most often found in *E. coli* and *K. pneumoniae*, however, they have also been found in *Proteus* spp., *Providencia* spp., and other genera of Enterobacteriaceae. The SHV-1 -lactamase is most commonly found in *K. pneumoniae* and responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species [15]. In many strains *Klebsiella pneumoniae*, blas$_{SHV-1}$ or a related gene is integrated into the

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bacterial chromosome[16]. The OXA-type enzymes are another growing family of ESBL. These -lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d[14]. The OXA-type lactamases confer resistance to ampicillin and cephalothin and is characterized by their high hydrolytic activity against oxacillin and cloxacillin.

Genes encoding ESBL are usually located on conjugative plasmids (such as bla_{TEM} or bla_{SHV}) although many of the most recently described ESBL genes are frequently found within integrons-like structures (such as bla_{CTX-M})[13,17-19]. Five integrons classes related to antibiotic resistance have been described based on the homology of their integrase genes [20-22,]. ESBL located on integrons-like structures are being increasingly reported worldwide[18-19].

The increasing resistance pattern to a drug creates pressure to switch to a different more potent drug as prescription. It is also mentionable that just as drug resistance is mainly an acquired property; it can also be lost in course of time. Therefore, vigilance is needed in screening the drug resistance pattern of different antibiotics which should be a continuous process. Antimicrobial resistance surveillance is necessary to determine the size of the problem and to guide empirical selection of antimicrobial agents for treating infected patients. The goal of this study was to determine the current prevalence of multidrug resistant and ESBL producing strains among UTI causing *E. coli* isolates against commonly used antimicrobial agents.

Materials and Methods

Sample collection site and time period

Ninety five cultures of *Escherichia coli* were isolated, identified and collected from UTI cases in Combined Millitary Hospital Dhaka between April, 2012 and July, 2012.

Selection of patients

Clinically diagnosed patients of Urinary tract infection (UTI) regardless of age, sex and socioeconomic status with *Escherichia coli* were included in this study.

Bacterial strains collection and short time storage

Clinically isolated individual colonies of *E. coli* from urinary tract infection cases were taken from McConkey agar plate (Urine Sample). These bacterial samples were inoculated in vials containing soft nutrient agar by stab inoculation. These vials were incubated at 37ºC (Memmert, Germany) for 18 hours and stored at 4ºC for short time storage overlaying with sterile mineral oil.

Antimicrobial agent susceptibility testing

Kirby-Bauer disk diffusion method was used to determine the antimicrobial agent sensitivity profiles of the *E. coli* isolates [23.24] for 14 antimicrobial agents belonging to 7 broad classes (namely penicillin, aminoglycoside, carbapenems, cefalosporins, monobactams, nitrofurans, and quinolones). These antimicrobial agents were chosen on the basis of their importance in treating human infections and on the basis of their ability to provide diversity for representation of different antimicrobial agent classes. Commercially available discs (Oxoid Limited, Hampshire, England) were used for the test.

Determination of ESBL production by double disc diffusion synergy method

One well isolated colony of the test isolate was incubated in 5 ml Muellar Hinton broth at 37°C and 250 rpm shaking for about 4 hours. When the optical density matched that of 0.5 McFarland turbidity standard (0.5 Macfarland standard represents 0.132 OD at 600 nm), the bacterial suspension was used to inoculate Mueller Hinton Agar (MHA) plates by swabbing them with sterile cotton swab. 30 mg discs of aztreonam, ceftazidime, ceftriaxone and cefotaxime were placed 15 mm (edge to edge) from an augmentin (amoxicillin-clavulanate; 20:10 mg) disc. Inoculated plates were incubated overnight at 37°C. Enhancement of the zone of inhibition between the clavulanate disc and any one of the β-lactum discs indicated the presence of an ESBL producing organism.

Isolation of plasmid DNA

Plasmid DNA isolation was carried out following the alkaline lysis method described by Birnbiom and Doly [25]. Five ml LB in a MacCartney bottle for each *E. coli* sample was prepared and autoclaved. One well isolated *E. coli* colony was added to a MacCartney tube and grown aerobically overnight at 37ºC in an orbital shaker (Memmert, Germany) at 250 r.p.m. Next day, before starting the next part of experiment, 95% ethanol was cooled in 20ºC freezer and solution III (3M CH_3COOK, pH adjusted to 4.0-4.8 with Glacial acetic acid) was cooled in ice. One ml of overnight culture was transferred to a 1.5 ml eppendorf tube and was centrifuge at 13,000 rpm for 5 minutes. The supernatant was discarded. The pellet was thoroughly suspended in 100 µl of solution I [50 mM Glucose, 25 mM Tris-HCl (pH: 8.0), 10 mM EDTA (pH: 8.0), Final pH 8.0 (Adjusted with HCl)] and was kept at room temperature for 5 minutes. 200 µl of solution II [Freshly prepared 1% (w/v) SDS, 0.2 N NaOH] was added and mixed gently by inverting the tube 5 times. It was kept on ice for 10 minutes. 150 µl of ice cold solution III was added mixed vigorously by vortexing for a few seconds. The tube was kept on ice for 5 minutes. The mixture was centrifuged for 10 minutes at 13,000 rpm to get the pellet of chromosomal DNA. About 450 µl of the clear supernatant containing the plasmid DNA was taken in a new Eppendorf tube. Two volumes (900 µl) of ice cold ethanol (95%) were added to each tube and vortexed for a few seconds to mix well. It was kept at room temperature for 10 minutes for DNA precipitation. The precipitated DNA was collected by centrifugation at 13,000 rpm for 10 minutes. The supernatant was discarded. To each tube, 1 ml 70% ethanol was added and vortexed well. It was centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded. The pellet was air dried at room temperature keeping the lid open or dried at 45ºC in a drier. The tube was flicked once/twice to check for any visible moisture still present. The dried DNA was dissolved in 50 µl TE buffer and was kept at 4ºC.

Isolation of genomic DNAby phenol-chloroform method

A 1.5 ml the overnight *E. coli* culture (grown in LB medium) was transferred to a 1.5 ml eppendorf tube and centrifuged at max speed for 1min to pellet the cells. The supernatant was discarded.

The supernatant was discarded as much as possible without disturbing the cell pellet. The cell pellet was resuspended in 600 ìl lysis buffer and vortex to completely resuspend cell pellet. The suspension was incubated for 1 hr at 37°C. An equal volume of phenol/chloroform mixture was added and mixed well by inverting the tube until the phases were completely mixed. Note: vortexing must be avoided as it can shear the DNA The rubes were spinned at max speed for 5 min at RT (all spins are performed at RT, unless indicated otherwise). A white layer (protein layer) at the aqueous phenol/chloroform interface was observed. The upper aqueous phase was carefully transferred to a new tube by using 1 ml pipetman (to avoid sucking the interface, 1 ml tip with wider mouth was used by cutting 1 ml tip-mouth about ~2 mm shorter). Steps 4-6 can be repeated until the white protein layer disappears. To remove phenol, an equal volume of chloroform was added to the aqueous layer. Again, it was mixed well by inverting the tube. The tubes were centrifuged at max speed for 5 min. The aqueous layer was transferred to a new tube. To precipitate the DNA, 2.5 or 3 volume of cold 200 proof ethanol (stored at -20°C freezer) was added and mixed gently (DNA precipitation was visible). The tube was then incubated at -20°C for 30 min or more. The tubes were centrifuged at max speed for 15 min at 4°C. The supernatant was discarded and the DNA pellet was rinsed with 1 ml 70% ethanol (stored at RT). The tubes were spinned at max speed for 2 min. The supernatant was discarded carefully and the DNA pellet was air-dried (The tube was tilted a little bit on paper towel). To be faster, the pellet can be dried at 37 °C incubator. The DNA pellet was resuspended in TE buffer. Note: lots of RNA is present in the DNA sample. So, for subsequent reaction, for example, to digest plasmid DNA, 1-5 ul (1 mg/ml) RNAase is added to the digestion solution to completely remove RNA. Or, RNAase is added directly to lysis buffer with a final concentration of 1mg/ml.

Separation of plasmid DNA by horizontal agarose gel electrophoresis

Electrophoresis was carried out in horizontal 0.8 % agarose gel and the method followed was as described by Meyers *et al* [26]. The gel was viewed on Gel Doc (BioRad).

Isolation of genomic DNA by phenol chlorophorm method

Genomic DNA was isolated by phenol-chlorophorm method and treated with RNAase.

Polymerase chain reaction (PCR)

Genomic DNA and plasmid DNA were both used separately as the template. For plasmid DNA, 1-10 ng and for genomic DNA, 50-100 ng DNA per reaction was used. Table 1 shows the bla_{SHV} and bla_{OXA} primers, $T_{Annealing}$ and their amplified product size.

Table 1: *Primers for the detection of bla_{OXA} and bla_{SHV} genes*

Gene encoding ESBL	Primer	Oligonucleotide sequence (5' to 3')	$T_{Annealing}$ (°C)	Size of amplified product (bp)
bla_{SHV}	SHV-F	5'CAC TCA AGG ATG TA TGT G3'	50	885
	SHV-R	5'TTA GCG TTG CCA GTG CTCG3'		
bla_{OXA}	OXA-F	5'ACC AGA TTC AAC TTTCAA 3'	45	598
	OXA-R	5'TCTT GGC TTTT ATGC TTG 3'		

Results

Distribution of UTI cases according to sex and age

Among the 95 diagnosed positive UTI cases, only 30 cases were from male and the rest 65 cases were from female. A variation of UTI distribution was observed among 95 positive UTI in respect of age. The relative prevalence of UTI according to different age groups is presented in Figure 1. It was observed that the prevalence was most in "19 to 45 age group". It was least in lower age group, but quite significant in older age group.

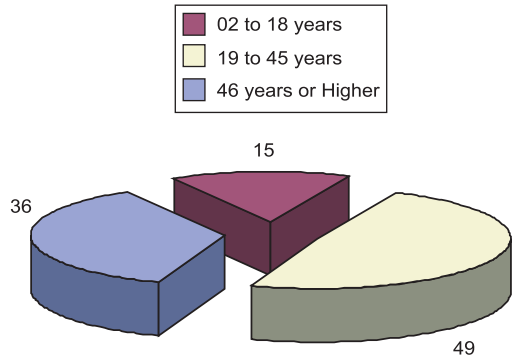

Figure 1: *Distribution of UTI cases according to age.*

Antibiogram of E. coli strains from UTI

All of the 95 *E. coli* strains were tested for antibiotic susceptibility. All of them were multi drug resistant (Figure 2). It was observed that 68% of the strains were resistant against 12 of the 14 antibiotic tested. More than 70 % samples were resistant to third generation cephalosporins. Resistance against ciprofloxacin, the frequently prescribed drug was seen to be 78%. Only nitrofurantoin and meropenem showed greater sensitivity towards the isolates showing 33% and 13% resistance respectively.

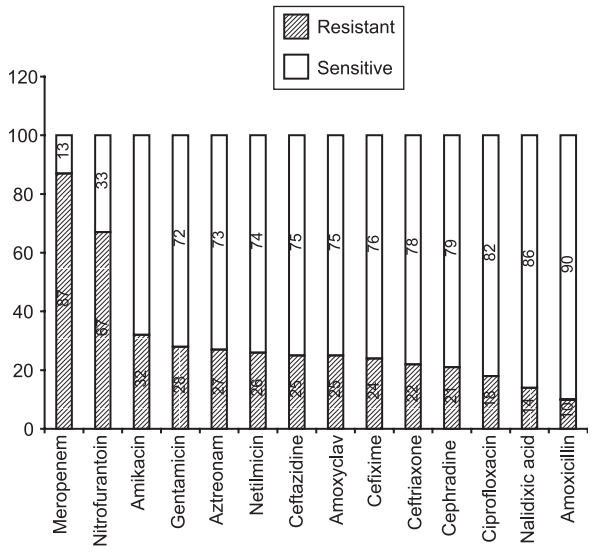

Figure 2: *Antibiotic susceptibility pattern of 95 MDR E. coli isolates from UTI.*

Detection of ESBL producing isolates by DDST (disk diffusion synergy test)

31 MDR *E. coli* isolates were randomly selected from the 95 isolates for the detection of ESBL Production by DDST. Out of these 31 isolates 21 (68 %) were found to be ESBL positive (Figure 3).

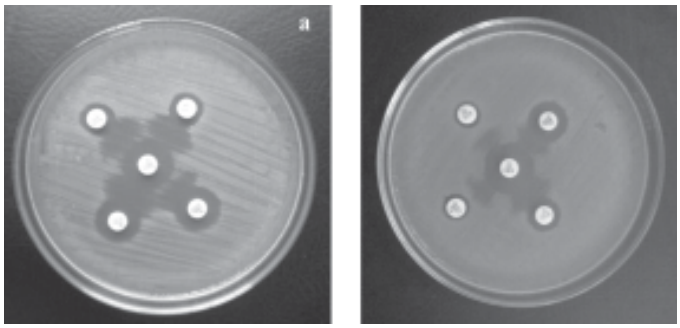

Figure 3 a, b: *Disk Diffusion Synergy Test of two of the isolates showing extended synergistic zones of inhibition*

Isolation of bla_{SHV} and bla_{OXA} genes by polymerase chain reaction method

PCR experiments were carried out using the 21 ESBL positive *E. coli* isolates. The PCR products were separated by agarose gel electrophoresis. All (100%) of the 21 isolates showed the 598 bp bla_{OXA} gene (Figure 5). Whereas, 8 (38%) isolates among the 21 showed the 885 bp bla_{SHV} gene (Figure 6).

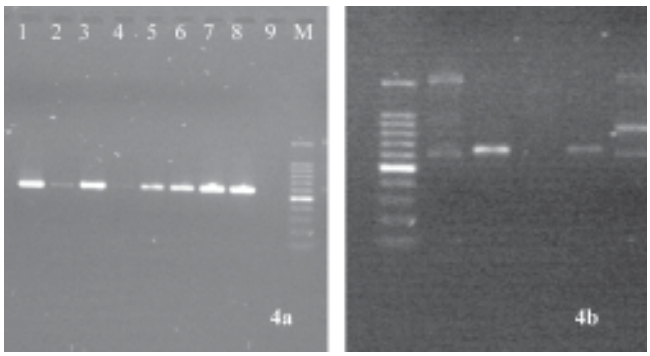

Figure 4: a. *Agarose gel electrophoresis showing the 598 bp bla_{OXA} gene. Lane 1-3 shows PCR products that used gDNA as template. While lanes 4-8 shows PCR products that used plasmid DNA as the template. Lane 9 shows reagent blank and M is the 100 bp DNA molecular weight marker. b. Agarose gel electrophoresis showing the 885 bp bla_{SHV} gene. Lane 5 showing the 885 bp fragment. M represents 100 bp DNA molecular marker. Some non specific amplification products are visible as well. This may be due to the non specific annealing of primers due to annealing temperature or ionic concentration of the reaction mix.*

Discussion

All of the multi drug resistant isolates of the present study showed a high degree of drug resistance to various commonly prescribed drugs. In total, 95 *E. coli* isolates from UTI regardless of sex and age of the patients were examined for antibiotic resistance with about 14 drugs. More than 70 % of the isolates were resistant to third generation cephalosporins. Resistance against ciprofloxacin, the frequently prescribed drug was seen to be 82 %. Similar results with ciprofloxacin resistance have been reported in another study carried out by Hassan *et. al*.[27]. Only meropenem and nitrofurantoin showed greater sensitivity towards the isolates showing 33% and 13% resistance respectively. High degree of resistance could be explained by the fact that these drugs are easily available in the pharmacy without doctor's prescription. Self-prescribed and relatively cheaper antibiotics used for all type of infection by patients, quakes and doctors and are often taken in inadequate doses resulting in high degree of resistance. Given that majority of therapy for UTI is empiric and that urinary tract pathogens are demonstrating increasing antibiotic resistance, continuously updated data on antimicrobial resistance patterns would be beneficial to guide empiric treatment.

During the second phase of the study, 31 isolates were randomly selected for ESBL production determination by double disc diffusion synergy test. Of these, 21 (67.74%) were found to be ESBL positive. This percentage is very high. However some other studies have reported similar or even higher percentage of ESBL producers among *E. coli* from UTI[3,27]. In a study carried out is Sweden, the number of patients with ESBL producing *E. coli* increased significantly from 5 to 47 per year[28-29]. In a study on enterobacteriaceae in Bangladesh, 71% of the 300 isolates were found to be ESBL positive by DDST method[30].

Extended-spectrum β-lactamases of the TEM-, SHV-, OXA-, and more recently, CTX-M-type enzymes have been described in many countries[31]. Although there is no systematic report on the ESBL-producers in Bangladesh, few study were carried out on the prevalence of the ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* that were isolated from Urban Hospital at Dhaka and from some regional medical colleges of Bangladesh. In which, 43.2 % *E. coli* and 39.5 % *Klebsiella pneumoniae* were the ESBL-producers[32]. In addition to the phenotypic characterization, the present study had focused particularly on the molecular characterization of the ESBL-producing *E. coli* isolates.

The 21 isolates with ESBL phenotypes were examined for the presence of $bla_{SHV,}$ and $bla_{OXA,}$ genes by PCR. All (100%) of the 21 isolated showed the 598 bp bla_{OXA} gene. Whereas, 8 (38%) isolates among the 21 showed the 885 bp bla_{SHV} gene (Figure 5,6). In the present study, both gDNA and plasmid DNA were used as the template DNA for PCR. This was done due to the fact that although ESBL genes are mainly carried on plsmids, they can reside on various integrons and can be found on gDNA as well. Both gDNA and plasmid DNA produced gene bla_{OXA} by PCR. However, comparatively few isolates produced $bla_{SHV,}$ gene

from both gDNA and plasmid DNA. Due to the unavialbility of primers for other ESBL genes, more experiments on other ESBL genes could not be carried out.

It has been argued that there is a direct relationship between the antibiotic used and the frequency and kind of antibiotic resistant strains in human being. This study highlights the needs for an antibiotic policy for its rationale use in our country. The policy should stress not only for prevention of infections, but also ensures proper selection of antibiotics and there should be minimum misuse of antibiotics. Clinicians must depend on more laboratory guidance, while laboratories must provide resistance pattern data for optimal patient management more rapidly. There is a need to better define strategies to prevent emergence.

Considering various findings of the present study, it can be concluded that extended spectrum beta lactamases are on the increase in Bangladesh. The co resistance to some other classes of antibiotics is very alarming. The study showed that only a limited number of drugs were sensitive for these bacteria and drug of choice according to the present study is imipenem, followed by nitrofurantoin and amikacin. Further studies are required to investigate MDR bacteria and ESBL from other parts of Bangladesh using more isolates. Studies of molecular epidemiology of these resistant genes can also be used for comparison with genes already isolated from other parts of the world.

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