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Original Article

Detection of New Delhi Metallo β Lactamase gene in Uropathogenic *E. coli*

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The rapid dissemination of antibiotic resistant *E. coli* is now a worldwide problem. In this study, a total of twenty *E. coli* obtained from stool were selected to determine resistance to beta lactam antibiotics. Beta–Lactamase are enzymes produced by bacteria that provide multi resistance to beta lactam antibiotics such as penicillin, cephalosporin, cephamycin and carbapenems. Of these isolates (n = 20), 35% were found resistant to Amoxicillin Clavulanate, 5% to Imipenem, 50% to Ceftriaxone and 75% to Ampicillin. PCR amplification confirmed the presence of the New Delhi beta-lactamase gene (bla_{NDM}) in one isolate (5%, n=20). Plasmids of variable sizes were found in all the isolates. Beta lactam antibiotics are now commonly used for the treatment of disease. Resistance of 50% of the isolates to Ceftriaxone is alarming as this indicates that an alternative drug may soon need to replace this antibiotic for successful treatment. The finding of this study is also of public health concern as plasmids were found in most isolates and these mobile genetic elements can be transferred among clinical bacteria, thereby disseminating antibiotic resistance further limiting treatment options.

Keywords: NDM β Lactamase, Uropathogen, *E. coli*, Antibiotic resistance

Introduction

Carbapenems are β lactam antibiotics that differ in their chemical structure from penicillins and cephalosporins. This class of antibiotics are now increasingly used owing to increased resistance to cephalosporins in *Enterobacteriaceae*[1]. Carbapenems have proven efficacy in severe infections due to ESBL producing bacteria[2]. The bacterial enzymes, Carbapenamases, typically inactivate most beta-lactam antibiotics and have become a cause of concern in the field of antibiotic resistance[3]. New Delhi metallo (NDM) beta-lactamase enzymes belong to the B1 class of beta-lactamasesand are unique among the carbapenameses[3]. Since its description, NDM has spread to many countries worldwide and is common in South Asia[3-5]. The present study was conducted to determine the presence of NDM, and hence Carbapenem resistance, in Uropathogenic *E. coli*.

Material and Method

Bacteria

A total of 20 *Escherichia coli* stored in the laboratory (Laboratory 206, Department of Microbiology, University of Dhaka, Bangladesh) repository were included in the study. The samples were urine samples collected from Medinova Diagnostic, Dhanmondi, Bangladesh.

Determination of the Antibiotic Resistance Pattern of the Target

The isolates were subjected to antimicrobial susceptibility testing by disk diffusion method as recommended by Clinical Laboratory Standard Institute[6], using commercial antimicrobial disks. The antibiotic disks used in this study were: Ampicillin (10), Imipenem (10), Amoxycillin-clavulanate, Ceftriaxone (30).The method described in CLSI (2017) was followed[6]. An inoculating needle was touched to a freshly grown, well isolated colony on TSA plate and then inoculated into 1 ml of Muller-Hinton Broth (MHB). The culture were then incubated in a shaker at 37°C for 4 hours to obtain the actively growing culture, equivalent to 0.5 McFarland standard (1.5x 10^8 CFU/mL). A sterile cotton swab was dipped into the standard suspension, excess broth was purged by pressing and rotating the swab firmly against the inside wall of the tube above the fluid. The swab was then streaked evenly in three directions over the entire surface of the agar plate to obtain a uniform inoculum. A final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for three to five minutes before the disks were applied. Antibiotic impregnated disks were then applied to the surface of the inoculated plates with sterile syringe needle. All disks were gently pressed down onto the agar with sterile forceps to ensure complete contact with the agar surface. Within 15 minutes after the disks were applied, the plates were inverted and placed in an incubator at 37°C. After overnight incubation, the plates were examined for zone of inhibition and the diameter of the zone of inhibition was measured to the nearest whole millimeter by a ruler. The zone diameters for individual antimicrobial agents was then translated into susceptible, intermediate, or resistant categories according to the CLSI guidelines (2017)[6].

Plasmid Profiling

Plasmid DNA was prepared using Invisorb Spin Plasmid Mini Two Plasmid extraction Kit (Invisorb, Germany).A single colony from a fresh overnight culture plate of the test bacteria was inoculated in 5 ml Muller-Hinton Broth and incubated at 37°C

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overnight. From this, 0.5mL of this fresh *E. coli* culture was transferred to a 1.5 mL microcentrifuge receiver tube. This was centrifuged at 13000 rpm for 1 minute and the supernatant was removed completely. The pellet was resuspended in 250 ¼L Solution. To this, 250 ¼L solution B was then added and mixed gently but thoroughly. This was followed by addition of 250 ¼L solution C, mixing by inverting the tube and centrifuged for 5 minutes at 13000 rpm. The clarified supernatant was transferred to a spin filter and incubated at room temperature for 1 minute. It was then centrifuged at 11000 rpm for 1 minute. The filtrate was discarded and 750 ¼L Wash Solution was added. It was then centrifuged at 11000 rpm for 1 minute. The filtrate was discarded and the microcentrifuge tube with the spin filter was further centrifuged for 3 minutes at 13000 rpm. The spin filter was placed on a new 1.5 mL Receiver Tube and 100 ¼L Elution Solution was added. It was incubated at room temperature for 5 minutes and centrifuged at 11000 rpm for 1 minute. The resulting filtrate is the plasmid extract.

Separation of Plasmid DNA by Agarose Gel Electrophoresis

Plasmid DNA was separated by horizontal electrophoresis in 0.8% agarose slab gels in a TAE buffer at room temperature at 80 volts for 1.5 hours. Ten ¼L of plasmid DNA solution was mixed with 2¼L of tracking dye and was loaded into the individual well of the gel. The gel was stained with Et-Br for 25 minutes and destained with distilled water for 5 minutes. DNA bands were visualized and photographed using Gel Documentation with UV trans-illuminator. The size of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel and was compared with the mobility of the known size marker. 1kbp DNA ladder (NEB, UK) was used as marker.

Detection of ²-lactamase genes by PCR Assay

Desired organisms were sub-cultured from stock of the controls and transformed isolates on Mueller-Hinton Agar and a single colony was taken and grown on Muller-Hinton Broth for plasmid extraction. Plasmid Extraction was performed on the subcultures and freshly grown bacteria to obtain Plasmid DNA to be used in PCR.PCR amplifies DNA targets using pairs of primers in reaction tubes. PCR reactions were run for different classes of beta-lactamases. Different primers were used for class A, B, C, and D. Primer used for various groups are given in Table 1 and PCR conditions in Table 2.

Electrophoretic Analysis of Amplified DNA Product

TAE or Loading Buffer (1 L of 50X buffer): It contains 242 g Tris base, 57.19 ml Glacial acetic acid, 100 ml of 0.5M Na-EDTA (pH 8.0) and water upto 1 L. Agarose was purchased from Invitrogen, USA. Staining Solution: Ethidium bromide was dissolved in (10 µg/¼1) sdH_2O, kept at 4° C and protected from light.DNA molecules were resolved electrophoretically in an agarose gel (1.2% w/v analytical grade agarose). Agarose 1.2 g was dissolved in IX TAE (100 ml) at the appropriate concentration by heating in the microwave and then the gel were poured into the tray. After solidification, 10 ¼L of PCR product was mixed with 2¼L of gel loading dye and loaded into the slots of the gel with the aid of a micropipette. 1X TAE buffer was used for electrophoresis. Then the gel was stained with staining solution containing ethidium-bromide for 30 minutes and de-stained with distilled water for 15 minutes. The Et-Br stained DNA bands were observed on a UV transilluminator (Gel Doc, Bio-Rad, USA). Photographs were taken using Gel Doc machine attached to a computer and bands were analyzed with "Quantity One" software. The appropriate sizes of the PCR product was estimated using the 1kB marker (NEB, UK).

Table1. *Primer used for various BLA_{NDM}*

Types of gene	Primers	Sequence (5'-3')	Amplicon Size (bp)	Annealing Temp (°C)	Reference
Bla_{NDM}	AmpC F AmpC R	5' -GGTTTGGCGATCTGGTTTTC-3' 5' -CGGAATGGCTCATCACGATC-3'	621	48 (45sec)	7

Table 2. *PCR Programs used for target DNA amplification*

Temp (°C)	95	95	55	68	68	4
Time (min)	5	0.5	0.75	0.5	10	10
Cycles	35					

Result and Discussion

Antibiotic Resistance Pattern of the Target Isolates

Antibiotic resistance is a burning issue in the field of medicine[8]. Beta lactam antibiotic resistance bacteria are a serious issue in present time. They have beta lactamase enzyme which attack beta lactam ring and develop resistance. Another mechanism by which Gram-negative bacteria acquire antibiotic resistance is through the transfer of antibiotic resistance genes via mobile genetic elements. The present study was undertaken to investigate the presence of NDM beta-lactamaseresistance gene(s) in uropathogenic *E. coli*.

The isolates were resistant to Ampicillin, Amoxycillin, Imipenem and Ceftriaxone to various extent. A total of 75% of the isolates were resistant to Ampicillin, 50% to Ceftriaxone, 35% to Amoxicillin Clavulanate, and 5% to Imipenem. A graphical presentation is shown in Figure 1.

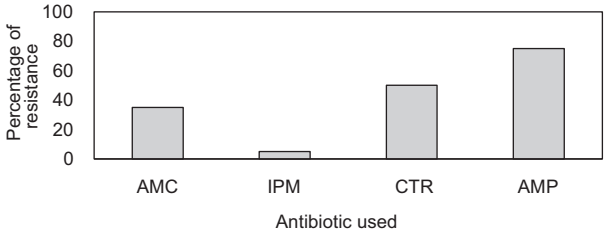

Figure1. *Pattern of resistance to ² lactam antibiotics*

The study was conducted with some clinical isolates of stool samples. Test isolates of *E. coli* exhibited different resistance patterns to different antibiotics. This could be attributed to treatment options prescribed, with isolates gaining resistance to the antibiotics they are most exposed to. In the present study, different isolates were resistant to ² – lactam antibiotics at various percentage. *Escherichia coli* is an important pathogen that shows increasing antimicrobial resistance in isolates from both animals and humans[9]. In a report by Ahsan and Islam (2019)[10], all isolates (collected from the same hospital as was done in our study) were found to be resistant to Amoxycillin (100%) compared to 75% in our study, and all except one were resistant to Amoxy-clav (97.2%) compared to 35% resistance to the same antibiotic in our study. A much lower percentage (5%) of resistance was observed to Imipenem in comparison to 72% in their study. In case of 3rd generation antibiotics, we found that 50% of our isolates were resistant to Ceftriaxone. This was in contrast to the findings of Ahsan and Islam (2019)[10], we found variable resistance of 71.7-97.8% resistant isolates in their study. In a previous report from Bangladesh, a large percentage of *E. coli* were resistant to at least three commonly used antibiotics[11]. One report from London shows that antibiotic resistance among community and nosocomial *E. coli* urinary tract isolates are most frequently seen against commonly prescribed antibiotics with Ampicillin (59.3%) and Trimethoprim (39.1%) being the least effective. The same report shows resistance of 12%, 9.3% and 8.3% against amoxicillin-clavulanate, ciprofloxacin and cephalexin[121]. Over the past few years, several reports have identified an increase in antibiotic resistance. However, the percentage resistance does vary between different regions. Resistance of *E. coli* from urine specimens in Switzerland to ciprofloxacin, trimethoprim/sulfamethoxazole, and amoxicillin/clavulanate had increased over the 1997 to 2007, from 1.8% to 15.9%, 17.4% to 21.3%, and 9.5% to 14.5%, respectively[12]. Similarly, a 30-year (1979–2009) follow-up study on *E. coli* in Sweden showed an increasing resistance trend for ampicillin, sulfonamide, trimethoprim, and gentamicin[13].

Plasmid Profile Analysis

Plasmid profile analysis of the 20 *E. coli* isolates was analyzed in 0.8% gel to understand the possible determinant of antibiotic resistance properties. In this study, the isolates showed the presence of plasmids of various sizes. Further tests were carried out later to detect the presence of ²-lactamase genes on these plasmids and to correlate the presence of these plasmids with antibiotic resistance. A representative gel showing plasmids extracted from the test isolates is shown in Figure 2.

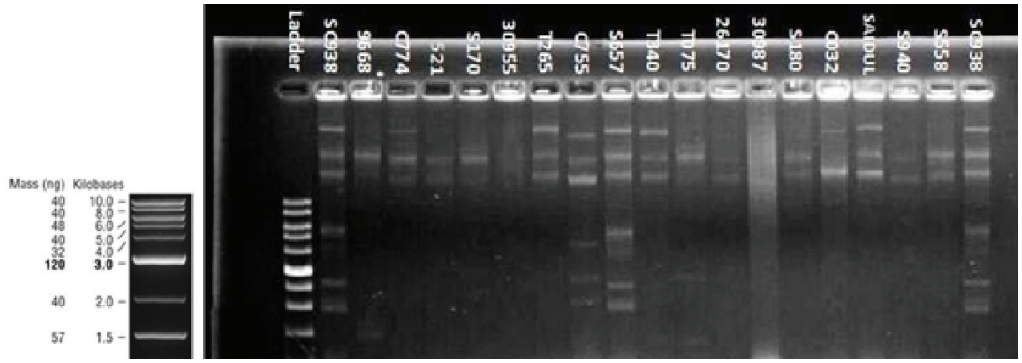

Figure 2. *Plasmid profile of isolates. The ladder is 1 kb plus DNA ladder from NEB detailed on the left.*

Bacterial resistance to beta- lactam antibiotics can be achieved by any of three strategies: the production of beta-lactam-hydrolyzing beta-lactamases enzymes, the utilization beta-lactam-insensitive cell wall transpeptidases, and the active expulsion of beta-lactam molecules from gram negative cells by way of efflux pumps. Determining the presence of plasmids in antibiotic resistant bacteria and establishing co-relation between presence of plasmid and antibiotic resistance is important because spread of resistance via plasmids can lead to outbreaks or endemic occurrence[14].

In the present study, plasmids varied in sizes from 1 kb to greater than 10 kb. In contrast, in our laboratory, we previously found plasmids ranging in sizes between less than one kb to about 7 kb. Prevalence of plasmids found in the present study was found to be different from previous findings[12, 15]. A similar study in our laboratory[10] could not find any correlation between Beta-lactam resistance and presence of plasmids. Production of ESBL is frequently reported to be plasmid encoded and bears clinical significance. Plasmids carrying ESBL genes may also carry genes for resistance to other antibiotics.

Analysis of New Delhi metallo ²-lactamase gene

We investigated for the presence of the NDM ²-lactamase gene by PCR and analyzed the amplicons in 1.5% agarose gel. Only one isolate harbored this gene. Screening of antibiotic resistance genes by plate-based assay is challenging[16]. Detection of specific genes by PCR and sequencing are usually conduced for the final confirmation of ESBL production. One of the mechanisms by which Gram-negative bacteria acquire resistance to beta-lactam antibiotics is through the production of beta-lactamases. Although ESBLs have been described in different Gram-negative bacteria, *Klebsiella pneumoniae* and *Escherichia coli* are the major ESBL-producing microorganisms worldwide[17-19]. In the present study, only one isolate (5%) harbored the New Delhi Metallo (NDM) ²-lactamase gene. In an earlier study in our laboratory on UPEC *E. coli* from the same hospital, 2.2% (n=66) isolates were MBL producers. Metallo Beta-Lactamases belong to class B[20] of Ambler's classification scheme. This class is further divided into subclasses B1, B2, and B3, of which Class-B1 enzymes are the most clinically significant[21-22]. New Delhi Metallo Beta Lactamase enzymes belong to Class B1 and confer resistance to all Beta-Lactam antibiotics known[23-24]. NDMs are no longer confined to India, Bangladesh or Pakistan[25-26]. NDM encoding genes are usually located on a readily transferable plasmid[27]. It was found that all of our *E.coli* contained multiple plasmids, which is in concordance with other findings. However, specific correlation between any plasmid and presence of NDM gene was not found.

The detection of antibiotic resistance profile of a pathogen is important, particularly in the context of treatment. The present study indicates the presence of multiple antibiotic resistant *E. coli* in clinical samples. The spread of these bacteria can lead to endemic outbreaks and the lack of awareness to treat these bacteria appropriately may confer resistance of these bacteria to more types of antibiotics. Preventive measures include only using antibiotics when needed, thereby stopping misuse of antibiotics or antimicrobials. Narrow- spectrum antibiotics are preferred over broad spectrum antibiotics when possible, as effectively and accurately targeting specific organism is less likely to cause resistance. For people who take medication at home, education about proper use is essential. The present study established the presence of ²-lactam antibiotic resistance genes in clinical *E. coli*, indicating great risk of spread of resistance through horizontal gene transfer (HGT) by these bacteria. Hence, care has to be taken against treating diseases involving them. Third generation ²-lactam antibiotics such as Ceftriaxone appear to lose their efficacy in treating *E. coli* infections and a point may be reached where treatment options may revert to first generation antibiotics such as Ampicillin. A fair assumption is that, if misuse and overuse of antibiotics in Bangladesh is not reduced then the effective antibiotics would not remain effective for long.

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