Comparison of Biofilm Producing and Non-producing *Escherichia coli* Isolated from Urine Samples of Patients Visiting a Tertiary Care Hospital of Morang, Nepal

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ABSTRACT

Objectives: The main objective of this study was to determine the prevalence of *Escherichia coli* among urinary tract infection (UTI) suspected patients visiting tertiary care hospital and to assess the biofilm producing ability of *E. coli* isolates.

Methods: A prospective cross-sectionalstudy was carried out in Biratnagar Metropolitan city, Eastern Nepal from December 2018 to May 2019. During the study 400 urine samples were collected from UTI suspected patients visiting a tertiary care hospital ofBiratnagar. Urine samples were cultured by using semi-quantitative culture technique and identified. Antibiotic susceptibility testing was done by Kirby-Bauer Disk Diffusion method according to CLSI (2011) guidelines.Biofilm assays were performed by microtitre plate method.

Results: This study reported 15% prevalence of *E. coli* out of 400 urine samples. 100% of *E. coli* isolates showed resistance to both Ampicillin and Amoxicillin while 100% were sensitive to Chloramphenicol. 70% (42/60) isolates wereMulti Drug Resistance (MDR)*E. coli*. The maximum isolates (86.66%) were found to be biofilm producers by microtitre plate method. Resistance to other antibiotics such as Nalidixic acid (71.11% vs 46.66%), Norfloxacin (53.33% vs 46.66%), Cotrimoxazole (42.22% vs 26.66%) was comparatively higher among biofilm producers than non-biofilm producers. There was a significance of association between biofilm and MDR ($p<0.05$).

Conclusion: There is relation between the ability of biofilm formation and drug resistance in the bacterium resulting to the failure of antibacterial drugs.

Key words: *E. coli,* Biofilm producer, Multidrug resistance (MDR), UTI

INTRODUCTION

Escherichia coli is Gram negative, facultative anaerobic and coliform bacterium which is common colonizer of lower intestine of warm-blooded animals (Tenaillon et al. 2012). Among all the members of *Enterobacteriaceae* family, *E. coli* is the most common pathogen (80-85%) involved in urinary tract infection (UTI) (Nicolle 2008; Bhatta et al. 2012). In case of UTI, fecal bacteria colonize urethra and spread up the urinary tract and finally to the bladder while sometimes to the kidneys causing pyelonephritis or the prostrate in males (Nicolle 2008).

During the lifetime approximately 10% of the humans acquire UTI at some time (Karki et al. 2004). The incidence of UTI is age and sex dependent example women are more prone to UTI than men (Nicolle 2008). Females falling within the age group 21-30 years experiences UTI more frequently (Baral et al. 2012).

Biofilm formation is a phenomenon which is produced by microorganisms to survive in harsh environment or for establishing bacterial infection in humans (Neupane et al. 2016). This protects bacteria from antibiotics and host defenses which as a result makes the treatment of infection more difficult (Anderson et al. 2003). The interaction between the bacterial cells within a biofilm can lead to the exchange of plasmid, drug resistance marker genes and hence enhances antimicrobial resistance (Watnick et al. 2000; Kostakioti et al. 2013). Thus, biofilm mode of living is advantageous for

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uropathogens to withstand stress and antibiotic drugs in urinary tract environment (Pramodhini et al. 2012).

According to the centers for disease control and prevention, multidrug resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al. 2011). The emergence of multidrug resistance *E. coli* in urinary tract infection has become a global concern (Mashwal et al. 2017). Study has reported *E. coli* being resistant against trimethoprim-sulfamethoxazole, fluoroquinolones and other antibiotics including ciprofloxacin (Karlowsky et al. 2006). The diagnosis of UTI is usually based on a quantitative urine culture yielding greater than 10^5 colony forming units per mL (Kass et al. 1957). However, several studies suggest that more than one third of symptomatic women show CFU counts below this level (low-coliform-count infection) and that a bacterial count of 100 CFU per mL of urine has a high positive predictive value for cystitis in symptomatic women (Komaroff et al. 1986; Kunin et al. 1993).The main aim of this study was to determine the prevalence of *E. coli* among UTI suspected patients visiting tertiary hospital and to assess the biofilm producing ability of *E. coli* isolates.

MATERIALS AND METHODS

Study site: This research was performed from December 2018 to May 2019 after receiving ethical approval from Nepal Health Research Council (NHRC), Kathmandu. During the study 400 urine samples were analyzed. All the works related to research were performed in microbiology laboratory of tertiary care hospital and of Central Campus of Technology, Dharan. The urine samples were taken from urinary tract infection suspected patients visiting a tertiary hospital of Biratnagar.

Sample collection: The midstream urine samples were collected from UTI suspected patients in sterilized screw-cap propylene bottles following standard guidelines (Isenberg 2004). The samples were then processed in microbiology laboratory as soon as after the collection. The containers were labeled with patient's name, ID number, specimen type and date of collection. In case of any delay in processing for more than 2 hours, samples were refrigerated at 4°C.

Isolation and identification: Urine specimens were cultured by using semi-quantitative culture technique as described by Kass (1962). A loopful of well-mixed sample was inoculated using standard calibrated loop onto Cystine-Lactose-Electrolyte-Deficient Agar (CLED) (HiMedia, India) and incubated aerobically at 37°C for 24 hours. After overnight incubation, colony counts yielding bacterial growth of $\geq 10^5$ were taken as being significant for UTI. For identification of isolates, at first colony characteristics of isolated bacteria were observed on agar plates and Gram staining was done. Gram negative isolates were then further identified by performing different biochemical tests including catalase, oxidase, indole utilization test, citrate test, methyl red, VP test, carbohydrate fermentation test and triple sugar iron utilization test. Isolates other than *E. coli*were not considered for this study.

Microtitre plate method for detection of biofilm: This method was performed as described by Borucki et al. (2003). Each culture was individually grown overnight in 10 mL of Trypticase Soy Broth (TSB) (HiMedia, India) at 37°C for 24 hours and diluted to 1:40 in TSB containing 0.25% glucose. Then 200µl of diluted culture was inoculated in a sterile microtitre well. The plates were incubated at 37°C for 24 hours for biofilm production. After incubation, content of each well was removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (PBS with pH 7.4) for four times and finally stained with 0.1% crystal violet solution for 30 minutes. After rinsing thrice with the sterile distilled water and subsequent drying, the stain taken up by the adherent biofilm was extracted by using 95% ethanol at 4°C. The content of each well was transferred to another microtitre well and the absorbance was measured at 595nm by ELISA plate reader(Loncare LR-620 microplate reader, Medical Technology Co., Ltd.).The experiment was performed in triplicate.Interpretation was made on OD by subtracting OD of control wells from OD of test wells. The optical density (OD_s) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (OD_{nc}). The following classification was used for the determination of biofilm formation: no biofilm production ($OD_s \leq OD_{nc}$), weak biofilm production ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm production ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$) and strong biofilm production ($4.OD_{nc} < OD_s$) (Stepanovic et al. 2007).

Antibiotic susceptibility test (AST): Antibiotic susceptibility of *E. coli* was evaluated against antibiotics ampicillin, chloramphenicol, sulfonamides, tetracycline,

ciprofloxacin, trimethoprim-sulfamethoxazole, cefotaxime and nalidixic acid by Kirby Bauer disc diffusion method following CLSI (2011) guidelines. Sub-cultured colonies were taken from nutrient agar plates and turbid suspension was made as per 0.5 McFarland standards by emulsifying colonial growth in Luria-Bertani broth (LB) (HiMedia, India). A sterile cotton swab was dipped into LB and the swab was streaked on the entire surface of Mueller Hinton agar (HiMedia, India) three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums. Finally, swab was done all around the edge of the agar surface. Using sterile tweezers, antibiotic discs were placed aseptically on the surface of Mueller Hinton agar plates. The plates were then incubated at 37°C for 24 hours.

Data analysis: The information was collected from questionnaire and finally tabulated. The data were analyzed by SPSS version 16. The p value less than equal to 0.05 was considered statistically significant.

RESULTS

Prevalence of *E. coli*

Out of 400 samples, 15% were positive for *E. coli*.

Table 1: Prevalence of *E. coli* in urine samples from UTI suspected patients

E. coli in urine samples		Prevalence	
Positive		60 (15%)	
Negative		340 (85%)	
Gender wise prevalence of *E. coli*			
Gender	**Number of subjects**	**UTI by *E coli***	**p-value**
Male	9	1(11.11%)	<0.05
Female	391	59(15.08%))	

Antibiotic susceptibility pattern of *E. coli* isolates

The most effective drugs for *E. coli* were found to be Chloramphenicol (100%), Cephoxitin (78.33%) and Ofloxacin (78.33%). *E. coli* were resistant to Ampicillin (100%), Amoxicillin (100%) and Nalidixic acid (65%).

Table 2: Antibiotic susceptibility pattern of *E. coli* isolates

Antibiotics	Resistant (%)	Sensitive (%)	p-value
Amoxicillin	60(100)	-	-
Ampicillin	60(100)	-	-
Cefotaxime	16(26.66)	44(73.33)	<0.001
Ceftriaxone	15(25)	45(75)	<0.001
Cephoxitin	13(21.66)	47(78.33)	<0.001
Chloramphenicol	-	60(100)	-
Ciprofloxacin	12(20)	48(60)	<0.001
Co-Trimoxazole	23(38.33)	37(61.66)	0.018
Gentamycin	14(23.33)	46(76.66)	<0.001
Nalidixic acid	39(65)	21(35)	0.01
Norfloxacin	31(51.66)	29(48.33)	0.584
Ofloxacin	13(21.66)	47(78.33)	<0.001
Tetracycline	26(43.33)	34(56.66)	0.201
Trimethoprim	22(36.66)	38(63.33)	0.060

Multidrug resistant (MDR) *E. coli*

Uropathogenic *E. coli* which showed resistance to three or more than three antibiotics were considered as multidrug resistant. 42 (70%) isolates of *E. coli* were MDR out of 60 isolates.

Table 3: Multidrug resistant (MDR) *E. coli*

Samples	Uropathogenic *E. coli*
Total samples	60
Multidrug resistant	42 (70%)

Antibiogram of biofilm producer and non-producer *E. coli*

Ampicillin and Amoxicillin were resisted by all isolates of *E. coli*. The biofilm producing *E. coli* showed high resistance to all antibiotics as compared to biofilm non-producer *E. coli*

Table 4: Antibiogram of biofilm producer and non-producer *E. coli*

Antibiotics	% of Biofilm Producing Resistant to antibiotics	% of non-biofilm Producing Resistant to antibiotics	P-value
Amoxicillin	100	100	-
Ampicillin	100	100	-
Cefotaxime	31.11	13.33	0.004
Ceftriaxone	31.11	6.66	0.001
Cephoxitin	24.44	13.33	0.019
Chloramphenicol	-	-	-
Ciprofloxacin	24.44	6.66	0.006
Cotrimoxazole	42.22	26.66	0.002
Gentamycin	28.88	6.66	0.002
Nalidixic acid	71.11	46.66	<0.001
Norfloxacin	53.33	46.66	0.002
Ofloxacin	24.44	13.33	0.019
Tetracycline	44.44	40	0.007
Trimethoprim	42.22	20	0.001

DISCUSSION

The overall prevalence of *E. coli* in urine samplesfrom UTI suspected patients was15% (60/400). Neupane et al. (2016) and Khatri et al. (2017) showed very similar report of 15.5% and 14.1% respectively. In this study the prevalence of UTI by *E. coli* was higher in female population than in male population which was statistically significant ($p<0.05$) which is consistent with many other studies.

In this study out of total 400 samples, 71 (17.77%) urine samples showed significant growth of uropathogens ($\geq 10^5$ cfu / mL) in which *E coli* was isolated from 60 (15%) urine samples Ponnusamy et al. (2012) and Sherchan et al. (2016) reported comparatively higher percentage of *E. coli* 23.49% and 87.9% of UTI casesrespectively. According to a research done by Neupane et al. (2016), 18.8% of the sample population showed significant growth of bacteria which is very similar to our result. All *E. coli* isolates were sensitive to Chloramphenicol and resistant to Amoxicillin and Ampicillin. A very close similarity was revealed by Sharma et al. (2013) and Ouno et al. (2013).

In our study, 70% *E. coli* were MDR. Baral et al. (2012) recorded 41.1% of MDR *E. coli* isolates in his investigation which was very less in comparison to our work. As per the experimentation done by Dehbanipour et al. (2016) and Poursina et al. (2018) multidrug resistant *E. coli* were73% and 68% respectively and it was very close to our analysis. Multidrug resistance has become a major problem in the treatment of diseases. The resistance of UTI causing bacteria towards commonly used antibiotics is escalating both in developing and developed countries (Elsayed et al. 2017).

Among 60 *E. coli* isolates, 31.66% were strong biofilm producers, 21.66% moderately positive, 21.66% were weak ones and 25% were biofilm non-producers by using microtitre plate method which were in accordance with the findings of Neupane et al. (2016) and Khatri et al. (2017).

Biofilm producing microorganisms shows resistance to large number of antibiotics increasing antibiotic resistance up to 1000 folds and hence, higher concentration of antimicrobial is required to treat such microorganisms (Stewart et al. 2001). Inadequate

amount of antibiotics reaching some areas of biofilm and inactiveness of bacteria located at the base of biofilm may be the reason for such resistance (Soto et al. 2014). In this investigation, both the biofilm producing and non-producing *E. coli* were resistant to Amoxicillin and Ampicillin (100%). However, resistance to other antibiotics such as Nalidixic acid, Norfloxacin and Cotrimoxazole was comparatively higher among biofilm producers than biofilm non-producers. Furthermore, this study there was a statistical significance ($p<0.05$) between biofilm formation and multidrug resistance (MDR) which was also reported by Murugan et al. (2011) and Kulkarni et al. (2018).

CONCLUSION

High prevalence of Multidrug resistant *E. coli* in UTI suspected patients alarms the need of prescribing antibiotics based only on culture and sensitivity reports. There is relation between the ability of biofilm formation and antibioticresistance in the bacterium resulting to the failure of antibacterial drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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