Antibiotic Resistance, Biofilm Formation and Sub-Inhibitory Hydrogen Peroxide Stimulation in Uropathogenic *Escherichia coli*

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ABSTRACT: Uropathogenic *Escherichia coli* (UPEC) is the most prevalent cause of urinary tract infections (UTIs). Biofilm formation and antibiotic resistance could be high among the causative agent. The purpose of this study was to determine antibiotic resistance, biofilm production, and biofilm-associated genes, *bcsA* and *csgD*, and sub-inhibitory hydrogen peroxide (H_2O_2) stimulation in UPEC for biofilm formation. A total of 71 UPEC were collected from a tertiary care hospital in Kathmandu and subjected to identify antibiotic susceptibility using Kirby-Bauer disk diffusion. The biofilm formation was assessed using microtiter culture plate method while pellicle formation was tested by a tube method. In representative 15 isolates based on biofilm-forming ability, *bcsA* and *csgD* were screened by conventional polymerase chain reaction, and treated with sub-lethal H_2O_2. The UPEC were found the most susceptible to meropenem (90.2%), and the least to ampicillin (11.3%) *in vitro* and 90.1% of them were multi-drug resistant (MDR). Most UPEC harbored biofilm-producing ability (97.2%), and could form pellicle at 37°C. Among representative 15 isolates, *csgD* was detected only among 10 isolates (66.67%) while *bcsA* gene was present in 13 isolates (86.67%). This study revealed that level of biofilm production elevated after sub-lethal H_2O_2 treatment ($P = .041$). These findings suggested that the pathogens are emerging as MDR. The biofilm production is high and the majority of selected strains contained *bcsA* and *csgD* genes. Pellicle formation test was suggestive to be an alternative qualitative method to screen biofilm production in UPEC. The sub-inhibitory concentration of H_2O_2 may contribute in increasing biofilm formation in UPEC.

KEYWORDS: Uropathogenic *E. coli*, biofilm, antibiotic resistance, *bcsA*, *csgD*

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Introduction

Uropathogenic *E. coli* (UPEC) strains are responsible for the majority of urinary tract infections.[1-3] UPEC infect the host via cell surface hydrophobicity (CSH), fimbriae, curli fibers, and the colanic capsule, facilitating the bacterial biofilm lifestyle, enhancing persistence and resistance to host innate immune factors, and antibiotic resistance.[4-6] Intracellular bacterial communities (IBCs) are formed when intracellular bacteria encase themselves on the bladder surface in a polysaccharide-rich matrix.[7] The development of IBCs in bladder epithelial cells involves several phases, including reversible to irreversible attachment, microcolony formation, and maturation.[8-10] Environmental factors such as immunological response, oxidative stress, predation, and other environmental pressures influence the production of the extracellular matrix, which is regulated by transcription factors.[11] The presence of terminal electron receptors in the urine, together with reduced oxygen stress in the bladder, supports the preferred development of *E. coli* biofilms.[12] The pellicle, which forms at the air-liquid interface and enables adhesion between bacteria and assembles to construct multicellular architectures, is a type of biofilm.[13] Polysaccharides are often involved in the establishment of productive cell-to-cell contacts that contribute to the formation of pellicles at liquid and solid interfaces such as clumping of cell aggregates in liquid cultures. This signifies UPEC can form pellicles *in vitro* in an air-water interface, indicating curli is important for the formation of this kind of biofilm. The switching between pellicle and biofilm during infection or survival in the natural environment is still unfamiliar.[14] Curli are amyloid fibers that participate in the generation of biofilms and aid in the adherence of bacteria to the human bladder.[1,15] In *E. coli*, the genes involved in curli production are arranged into 2 operons: *csgAB* and *csgDEFG*. The *csgAB* encodes 2 curli components (*cgsA* and *csgB*), while *csgDEFG* is in charge of control, assembly, and transportation.[16] In the bacteria, the master regulatory gene *csgD* stimulates the production of curli and extracellular matrix.[17] The biofilm generated by Enterobacteriaceae contains cellulose as a major component.[18] In bacterial biofilms, cellulose acts as a structural component that contributes as a scaffold for biofilm formation.[19] The *bcsABZC* operon contains structural genes for cellulose expression.[20] The cellulose synthase enzyme is transcribed by the *bcsA* gene, and the transcriptional regulator *csgD* is connected to the regulation of cellulose production.[18,21]

Increasing antibiotic resistance against UTIs in recent years is emerging to be troublesome, which implies a serious threat to human health.[2] Antibiotic-resistant bacteria and their propagation in various settings have evidently become a major concern around the world.[22] The biofilm-forming isolates are resistant to antibiotic therapy, posing a major clinical concern in the case of biofilm-related infections.[23,24] Biofilm acts as a protective layer around bacteria, preventing antibiotics, immune cells, and host proteins from proliferating.[25] Various investigations among UPEC have shown that the production of biofilm is closely linked to antibiotic resistance and MDR.[26-28] However, some reports have demonstrated that resistance is not dependent on the production of biofilm.[29-32]

Reactive oxygen intermediates such as hydrogen peroxide (H_2O_2) are toxic molecules produced by immune cells in response to bacterial invasion into the host. Bacteria try to protect themselves against the immune system through specific properties such as biofilm formation. This phenomenon occurs also during urinary tract infections.[33] The bacterial biofilm is integral to many infections by promoting persistence, protecting from host innate immune factors, and resisting to antibiotics.[5,6]

Despite the fact that UPEC has been widely reported from clinical samples in Nepal, no study has yet reported the presence of *bcsA* and *csgD* genes in the UPEC for biofilm formation across the country. It is important to determine anti-microbial resistance for evaluating the effectiveness of the drugs. Thus, this study attempts to demonstrate antibiotic, and multi-drug resistance status in the pathogens. This research was also hypothesized upon, H_2O_2 under sub-inhibitory concentration stimulates biofilm production among UPEC and presence of *bcsA* and *cgsD* genes is associated with biofilm formation. The findings of this research are anticipated to provide new insights associated with the pathogenicity of the biofilm-producing UPEC.

Materials and Methods

Bacterial strains collection

In Bharosa Hospital, the urine samples were cultured on MacConkey agar (Hi-Media Laboratories Pvt. Ltd., India) and Blood agar (Hi-Media Laboratories Pvt. Ltd., India). The study period ranged from February 2019 to February 2020. The ethical approval from the Institutional Review Committee, Institute of Science and Technology was obtained for the research (IRC/IOST-Regd. No. 1).

A loopful of urine was streaked on the plates and then incubated at 37°C overnight. Colony count was performed to calculate the number of CFU per mL of urine and the bacterial count was reported as insignificant growth for 10^4 CFU/mL of organisms, 10^4-10^5 CFU/mL of organisms as doubtful, and significant bacteriuria was defined when the bacterial colony is more than 10^5 CFU/mL organisms.[34] The identification of *E. coli* was done by standard laboratory procedures. Gram staining was performed. Identification was carried out by various tests such as positive catalase test, negative oxidase test, motile, indole positive, citrate negative, urea hydrolysis test positive, fermentative in Hugh's and Leifson's medium, and TSI (triple sugar iron) test is with A/A with gas production.[35]

Antibiotics susceptibility testing

The confirmed isolates recovered from urine samples were subjected to antibiotic susceptibility testing (Kirby-Bauer disk diffusion) using Mueller Hinton Agar (Hi-Media Laboratories Pvt. Ltd., India). Altogether, 10 antibiotics (recommended by CLSI guideline 2020) were used which included ampicillin (10 µg), ciprofloxacin (5 µg), cefalexin (30 µg), cefepime (30 µg), ceftriaxone (30 µg), amoxyclav (30 µg), co-trimoxazole (25 µg), nitrofurantoin (300 µg), gentamicin (10 µg), and meropenem (10 µg) (Hi-Media Laboratories Pvt. Ltd., India). The pathogens were categorized as resistant and sensitive. Those bacteria were considered as MDR strains when they were found non-susceptible to at least one agent in 3 or more antimicrobial categories.[36]

Pellicle test

The isolates were grown without shaking which included overnight incubation in 5 mL Luria broth (LB) at 37°C and transferred into 4 mL LB in 15 mL glass tubes. After 48 hours at 37°C, the formation of the pellicle at the air-liquid interface was visually observed.[37]

Biofilm assay

In this study, 71 isolates were employed for the quantitative test of biofilm as described by Christensen et al.[38] A loopful of test organisms isolated from fresh agar plates were inoculated in 1 mL of tryptone soya broth (TSB) (Hi-Media Laboratories Pvt. Ltd., India) with 1% glucose. Broths were incubated at 37°C for 24 hours which were then diluted at 1:100 with fresh TSB at 100 rpm. Then, 96 well microtiter plate was filled with 200 µL of diluted culture broth in each well and incubated for 48 hours. TSB with 1% glucose was used as the negative control in 1 lane of the microtiter plate and *E. coli* (ATCC 25922) as a positive control in another 3 wells. After the incubation, the contents of each well were removed by gentle tapping. The wells were then washed with 0.2 mL phosphate-buffered saline (pH 7.3) 4 times to remove the free-floating bacteria. The biofilm formed by the bacteria adherent to the wells were fixed by 2% sodium acetate and then stained by 100 µL of 0.1% crystal violet for 15 minutes at room temperature. Excess stain was removed with deionized water and the biofilm was quantified by measuring the absorbance at 630 nm against a blank in Multiskan Sky/Microtiter spectrophotometer (Thermo Fisher Scientific, USA) equipped with SkanIt software version 5.0. following solubilization of attached biofilm in 95% ethanol.[39] The experiment was performed in triplicate and repeated 3 times. The interpretation of biofilm production

Table 1. Comparison of antibiotic susceptibility to biofilm producers in the non-biofilm forming environment (n = 71).

ANTIBIOTICS	SENSITIVE		RESISTANT		TOTAL RESISTANT-UPEC (%)	*P*-VALUE
	BIOFILM PRODUCERS (%)	BIOFILM NON-PRODUCERS (%)	BIOFILM PRODUCERS (%)	BIOFILM NON-PRODUCERS (%)		
Ampicillin (AMP)	8 (11.3)	-	61 (85.9)	2 (2.8)	63 (88.7)	.6
Amoxyclav (CAC)	48 (67.6)	1 (1.4)	21 (29.6)	1 (1.4)	22 (31)	.56
Ciprofloxacin (CIP)	41 (57.8)	1 (1.4)	28 (39.4)	1 (1.4)	29 (40.8)	.79
Cefalexin (CN)	48 (67.6)	-	21 (29.7)	2 (2.8)	23 (32.4)	.038
Co-Trimoxazole (COT)	18 (25.4)	1 (1.4)	51 (71.8)	1 (1.4)	52 (73.2)	.46
Cefepime (CPM)	46 (64.8)	1 (1.4)	23 (32.4)	1 (1.4)	24 (33.8)	.62
Ceftriaxone (CTR)	48 (67.5)	2 (2.8)	21 (29.7)	-	21 (29.7)	.56
Gentamicin (GEN)	54 (76.1)	2 (2.8)	15 (21.1)	-	15 (21.1)	.46
Meropenem (MRP)	62 (87.4)	2 (2.8)	7 (9.8)	-	7 (9.8)	.6
Nitrofurantoin (NIT)	55 (78.9)	2 (2.8)	14 (18.3)	-	14 (18.3)	.042

Biofilm-formation is non-significant ($P > .05$) with AMP, CAC, CIP, COT, CPM, CTR, GEN, and MRP and biofilm-formation is significant ($P < .05$) with CN and NIT on MHA.

was done according to the criteria of Stepanović et al.[40] The cut-off optical density (ODc) is defined as 3 standard deviations above the mean OD of the negative control.

Detection of bcsA and csgD

Only 15 isolates were subjected to a polymerase chain reaction, which includes 3 strong producers, 5 moderate producers, 5 weak producers, and 2 non-biofilm producers regarding biofilm formation. The primers used for amplifying *bcsA* (base pair 826 bp) were F: GCTTCTCGGCGCTAATGTTG and R: GAGGTATAGCCACGACGGTG[41] and for *csgD* (base pair 97 bp) were F: CCGCTTGTGTCCGGTTTT and R: GAGATCGCTCGTTCGTTGTTC.[42] PCR for *bcsA* gene was done in a DNA thermal cycler (Applied biosystems, USA) with the setting: initial denaturation for 10 minutes at 95°C, followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute 30 seconds at 55°C and extension for 1 minute at 72°C, and a final extension for 10 minutes at 72°C.[41] PCR for *csgD* gene was done in the cycler with the setting: initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 57°C and extension for 1 minute at 72°C and a final extension for 10 minutes at 72°C. Electrophoresis was performed in 2.5% gel. Bromphenol blue was employed for loading DNA samples into agarose gel wells as well as tracking migration during electrophoresis.[42]

Treatment of bacterial strains with H_2O_2

Those selected 15 *E. coli* strains were cultured (10^6 CFU/mL) in Luria Bertani broth at 37°C for 24 hours along 0.625 mM H_2O_2, sub-inhibitory concentration for bacterial growth and crude catalase was added to stop the reaction after 15 minutes treatment with H_2O_2.[33] The source of catalase was *Solanum tuberosum*.[43] Then, determination of absorbance for biofilm for the treated strains was performed as described in the microtiter plate culture method.

Statistical analysis

All data obtained were analyzed using the statistical program statistical package for social science (SPSS v. 22.0) and OriginPro v. 8.5 for descriptive statistics. Different percentages, chi-square test (antibiotics, biofilm, pellicle, AST, MDR), and *t*-test (biofilm formation and biofilm-forming genes), chi-square test (association of *bcsA* and *csgD* with biofilm) were used to compare groups, and *P*-values $< .05$ were considered statistically significant.

Results

UPEC show the highest susceptibility to meropenem and the least susceptibility to ampicillin in vitro

Through the Kirby-Bauer test susceptibility method, the sensitivity of UPEC (n = 71) toward antibiotics was determined. The susceptibility pattern of UPEC isolates to different antimicrobial agents is shown in Table 1. Among the antibiotics, the bacterial resistance was extensively high toward ampicillin (88.7%) followed by cotrimoxazole (73.2%) and ciprofloxacin (40.8%). The isolates showed the least resistance toward meropenem (9.8%) followed by nitrofurantoin (18.3%) and gentamicin (21.1%). Altogether, 64 (90.1%) isolates were multidrug-resistant.

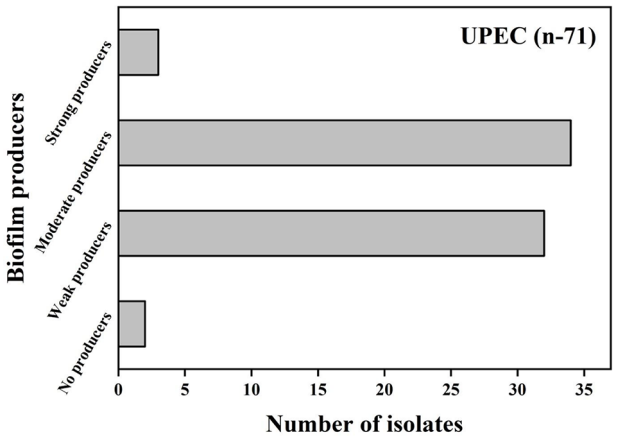


Figure 1. Biofilm production in UPEC via microtiter plate assay (n = 71).

UPEC demonstrate pellicle formation in air–liquid interface and corresponds biofilm formation ability at 37°C

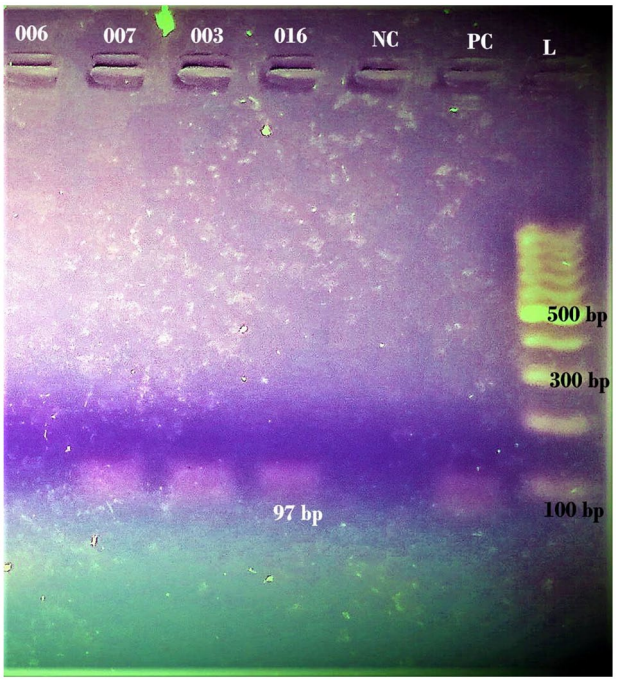
In the test tubes with Luria Bertani broth, 67 (94.34%) isolates were capable of forming pellicles in the air-liquid interface. Through microtiter plate culture assay based on the optical density of negative control, biofilm formation was categorized into 4 groups (Figure 1). The cut-off value of optical density for biofilm production was 0.062 obtained by adding 3 Standard Deviations to the value of negative control. The biofilm formation was observed in 69 (97.1%) isolates. Strong biofilm formation was observed among 3 (4.2%) isolates, weak biofilm formation among 32 (45.1%) isolates, moderate biofilm formation among 34 (47.9%) isolates, and 2 (2.8%) isolates were unable to form biofilm. Furthermore, there was a significant relationship ($P = .002$) between pellicle formation inside the tube and biofilm formation in the microtiter plate.

Cephalexin and nitrofurantoin are effective against biofilm-forming UPEC in a non-biofilm forming environment

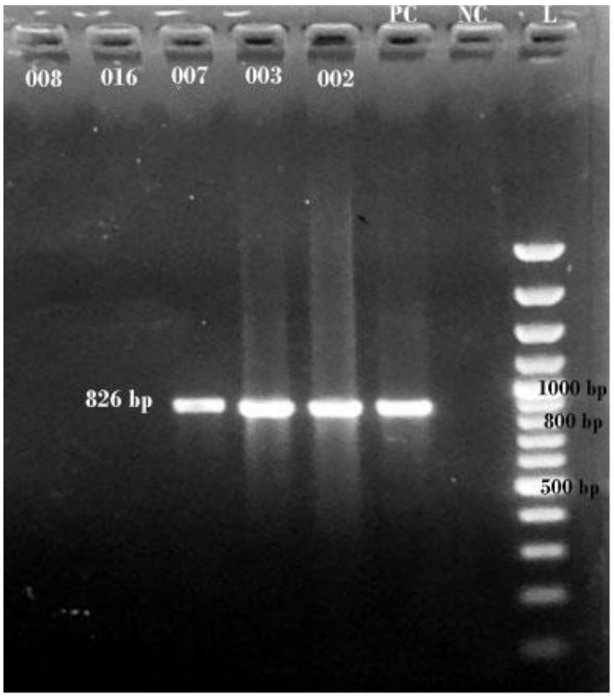
Comparing antibiotic susceptibility (AST test on MHA) and biofilm formation (Table 1), most of those selected antibiotics showed an insignificant relationship. Against ampicillin, amoxyclav, ciprofloxacin, co-trimoxazole, cefepime, ceftriaxone, gentamicin, and meropenem, the *P*-value was ⩾.05 which signifies biofilm-formation may not have a relation with these antibiotics used *in vitro* in non-biofilm-forming environment. Biofilm-forming bacteria in a non-biofilm-forming conditions appeared to be inhibited by administration of cephalexin ($P = .038$) and nitrofurantoin ($P = .042$) *in vitro*.

Co-occurrence of bcsA and csgD in biofilm production is significant

Detection of genes was carried out through conventional PCR, agarose gel electrophoresis, and visualization (Photograph 1) in a UV chamber. Among the 71 isolates, only 15 selected isolates were selected based on biofilm-forming ability. The gene, *csgD* was detected only among 10 isolates (66.67%)



Photograph 1(A). 2.5% agarose gel electrophoresis for detection of *csgD* (97 bp) in UPEC isolates (L = Thermo Scientific GeneRuler 100 bp plus DNA ladder).



Photograph 1(B). 1% agarose gel electrophoresis for detection of *bcsA* (826 bp) in UPEC isolates (*L* = Thermo Scientific GeneRuler 100 bp plus DNA ladder).

Table 2. Association of *bcsA* gene and *csgD* gene with biofilm.

GENOTYPE	BIOFILM		TOTAL	CHI-SQUARE TEST (*P*-VALUE)
	PRODUCERS	NON-PRODUCERS		
bcsA⁻*csgD*⁻	1	1	2	.236
bcsA⁺*csgD*⁺	9	1	10	
bcsA⁺*csgD*⁻	3	0	3	
Total	13	2	15	

bcsA ⁻ (*bcsA* absent), *bcsA*⁺ (*bcsA* present), *csgD* ⁻ (*csgD* absent), *csgD* ⁺ (*csgD* present).

Photograph 2. Hydrogen peroxide and catalase treatment of UPEC (Luria Bertani broths with hydrogen peroxide + crude catalase).

while *bcsA* gene was detected among 13 isolates (86.67%). The co-occurrence of *bcsA* and *csgD* in the isolates was significant ($P = .032$). Out of the total 13 producers; 12 (92.3%) producers harbored *bcsA* while 10 producers (76.92%) contained *csgD*. However, the association of *bcsA* with biofilm ($P = .116$) and *csgD* with biofilm ($P = .257$) were insignificant for both genes (Table 2). Detection of targeted genotypes (*bcsA* and *csgD*) among those UPEC indicates insignificance in biofilm ($P = .24$). Among 2 non-biofilm-producers, one isolate harbored both *bcsA* and *csgD* while the other isolate lacked both genes.

Sub-inhibitory H_2O_2 treatment elevated biofilm formation

The level of biofilm formation was measured based on optical density. The level of biofilm production increased after the treatment with sub-inhibitory H_2O_2, and catalase in non-producers, weak producers, and moderate producers (Photograph 2). In contrast, among the strong producers, the amount of biofilm formed was decreased (Table 3). The relationship between before treatment and after treatment is significant that is, *P*-value ≤ 0.05 which implies hydrogen peroxide can stimulate biofilm formation in the UPEC.

Discussion

Different studies display variability in the spectrum and frequency of antibiotic resistance among UPEC.[44-49] Our study showed the highest sensitivity toward meropenem since only 9.8% of UPEC are resistant to the antibiotic, which was similar to some studies.[49-51] Carbapenems are highly active against *E. coli* isolates and represent the best treatment option.[2,52] The highest resistance was observed with ampicillin (88.7%). Similar bacterial resistance to ampicillin was demonstrated in various studies.[48,49,53] Ampicillin was used as empirical therapy for a long time, and resistance may have emerged as a result of self-medication, increased antibiotic intake, and the emergence of resistant isolates.[47,54,55] In a research conducted by Yadav and Prakash in Southern Terai of Nepal, it was found that 91.86% of the isolates were MDR.[56] The results were similar regarding MDR rates.

The majority of isolates in our study showed pellicle formation. Nascimento et al conducted an investigation in which pellicle production in clinical isolates of atypical enteropathogenic *E. coli* was demonstrated (aEPEC).[37] Pellicles are also known as air-liquid (A-L) biofilms because they form at the air-liquid interface.[57] Pellicle development begins with bacteria adhering to the culture device's wall at the air-liquid contact, followed by the development of a monolayer by attached cells, and finally the formation of the distinctive three-dimensional architecture.[58,59] The pellicle is considered a special structure of biofilm.[60-62]

The formation of pellicle at the air-liquid interface was successfully observed among the isolates. At 37°C, the pathogen pellicles correspond to the ability to produce biofilm. A meta-analysis showed more than 84% of UPEC have the ability to form a biofilm.[28] Variation in the level of biofilm production was observed in different researches.[27,48,63,64] The study data depends upon the biofilm formation ability of the isolates determined by specific factors such as hydrophobicity, and cellular surface electrical discharge and varies among strains.[65] Likewise, the organisms tend to produce more biofilm to establish successful infection, biofilms are formed on urinary catheters or on/within bladder epithelial cells protecting them from the host immune system, antimicrobial therapy, and various dynamic environmental conditions.[12] According to our findings, pellicle formation can be used to screen for biofilm formation. Exopolysaccharides

Table 3. Effect of hydrogen peroxide (H_2O_2) treatment on biofilm production.

BIOFILM PRODUCTION LEVEL (SAMPLE CODE)	OPTICAL DENSITY BEFORE H_2O_2	OPTICAL DENSITY H_2O_2		*P*-VALUE
		ONLY H_2O_2	H_2O_2 + CATALASE (STIMULATION)	
Non-producer				
008	0.062	0.03	0.17	
016	0.049	0.04	0.22	
Weak				
003	0.09	0.06	0.61	
006	0.09	0.06	0.28	
007	0.07	0.05	0.20	
009	0.07	0.05	0.29	
015	0.08	0.04	0.29	
Moderate				.041
001	0.17	0.10	0.41	
004	0.14	0.09	0.45	
010	0.15	0.08	0.29	
011	0.19	0.10	0.37	
012	0.14	0.07	0.29	
Strong				
002	0.63	0.12	0.27	
005	0.33	0.10	0.22	
014	0.62	0.12	0.39	

are believed to be associated with the production of productive cell-to-cell interactions that contribute to the formation of biofilm communities at liquid and solid interfaces, such as clumping of cell aggregates in liquid cultures, according to a study.[66] The primary components for producing the pellicle matrix include oxygen, flagellar motility, and cellulose.[67-70]

Biofilm-forming bacteria are a prevalent cause of recurring and complex UTIs.[6] Biofilm-associated microorganisms are considered to be more resistant to antimicrobial treatments.[71] In this study, biofilm producers are more susceptible to cefalexin and nitrofurantoin. The susceptibility test showed cefalexin to be the most effective antibiotic against biofilm producers in non-biofilm forming conditions which was peculiar. Cefalexin displayed low sensitivity against bacteria in another finding.[56] However, based on geographical and regional location, antimicrobial sensitivity can vary.[72] A meta-analysis showed nitrofurantoin is the best antibiotic for invading UPEC strains.[28] According to a study by Makled et al, nitrofurantoin could be considered as selective antibiotics against biofilm structures.[73] Also, our finding suggests that nitrofurantoin was also effective against most of the biofilm producers. The findings could aid in the treatment of initial infection when biofilm is not formed in the isolates. Cefalexin and nitrofurantoin are frequently administered in the context of Nepal currently. Individual associations with resistance in *E. coli* to gentamicin and ceftazidime were seen in a research.[32] Antibiotic resistance can develop as a result of the synthesis of the ß-lactamase enzyme, the efflux pump, and decreased antibiotic uptake due to alterations in the outer membrane porin protein.[47] Furthermore, antibiotic tolerance is mediated through genetic changes at the bacterial chromosomal level.[74] Despite the fact that various integers suggest a link between antibiotic resistance and biofilm, this study found the contrary. As a result, more advanced research on uroepithelial organoids is needed to investigate the molecular links between antibiotic resistance and biofilm.

Our research revealed that *bcsA* was detected in more isolates than *csgD*. The expression of the *bcsA* gene, which codes for cellulose, had previously been linked to the csg operon, which also codes for curli fimbriae.[75] However, when comparing biofilm formation *in vitro*, our investigation found that *bcsA* was present in more isolates than *csgD*. The *bcsA* gene is not necessarily needed for biofilm formation in Enterobacteriaceae because other genes,

such as *csgD*, *adrA*, and other factors, can also be involved in cellulose expression and regulation.[76,77] There could be *csgD* independent pathway for cellulose formation.[77] In UPEC, cyclic AMP (cAMP) is also responsible for regulating curli and cellulose.[11] According to reports, the *pgaABCD* locus found in *E. coli* is required for biofilm formation.[48,78,79] In another investigation, the virulence genes *fimH*, *pap*, *afa*, and *sfa* were found to be strongly associated with biofilm formation.[80] However, Davari Abad et al could not ascertain the connection of biofilm formation with *sfa* and *afa* genes.[81] A significant correlation was established between biofilm production and the *sdiA*, *rcsA*, and *rpoS* genes.[82] These findings, combined with our own, reveal that biofilm formation is a complicated process that will require more research to understand the genetic makeup of biofilm formation.

Our finding suggests that hydrogen peroxide can enhance the biofilm-forming ability among non- producers, weak, and moderate biofilm producers despite the presence or absence *bcsA* and *csgD*. The level of biofilm production among the strong producers has been decreased. It may be due to exogenous quorum sensing inhibitor when binds with QS receptor inhibit the signaling and fail to produce further biofilm or the catabolite repression by glucose.[83]

So far, no investigations have been published in Nepal reporting the detection of *bcsA* and *csgD* genes in UPEC isolated from clinical settings. The limitations of the study were the antibiotic susceptibility test (AST) was not performed in the biofilm-forming environment and gene expression was not carried out. All samples were not included in the molecular study since the resources were limited. Only 15 isolates were screened for the target genes and sub-inhibitory H_2O_2 treatment. There are significant drawbacks to this study, such as it was limited to a single hospital, the short period of the study, and the use of crude catalase extract. To expand about epidemiological or virulence aspects of UPEC, the presence of *bcsA* and *csgD* genes could be checked among MDR and XDR biofilm forming strains by in-silico analysis of genomes in further studies. Our study may help researchers in accounting the virulence factor, and multi-drug resistance of the bacteria for developing further treatment strategies.

Conclusion

The effectiveness of meropenem against the isolates was demonstrated to be the highest. About 90% of the pathogens were MDR which indicates alarming threat to public health. The biofilm production was observed in more than 95% isolates. The pellicle formation test appeared to be a potentially viable qualitative approach for detecting biofilm formation as the ability of UPEC to form pellicles at 37°C correlates to biofilm formation capability. Cefalexin and nitrofurantoin were screened to be selective against UPEC capable of forming biofilm in non-biofilm forming conditions. The *bcsA* and *csgD* genes were found in the majority of the chosen strains. The sub-lethal dosage of H_2O_2 may contribute in elevating biofilm forming capacity in UPEC except in strong producers. Further researches must be warranted to encounter the research gaps in biofilm, antibiotic susceptibility, and response toward environmental stress in UPEC.

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Author Contributions

PD conceived the study design. PD and DRJ contributed to the design. PD planned the study and carried out the experiments and data analysis. AD guided in collecting and processing the samples in the hospital. BLM assisted in the laboratory for conducting the experiments. SanK, SudK, RT, AD, and DRJ guided in the interpretation, and manuscript writing. PD drafted the original manuscript. The manuscript was revised and edited by TPJ, DRJ and PD. All the authors contributed to the article and approved the submitted version.

Availability of Data and Material

All data collected in the study have been presented in the manuscript.

Code Availability

Not applicable

Consent to Participate

Written consent was obtained from the patients before the collection of samples and data.

Consent for Publication

Not applicable

Ethical Approval

Ethical approval was obtained from Institutional Review Committee of Institute of Science and Technology, Tribhuvan University, Kirtipur, Kathmandu (IRC/IOST-Regd. No. 1).

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