Biofilm Formation, Inhibition and Multi Drug Resistance in *Escherichia coli* Isolated from Clinical Specimens

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ABSTRACT

Objectives: *T*his study aimed to determine the prevalence of biofilm formation, assess the inhibitiory action of EDTA on biofilm production and to asses the antimicrobial resistance profiles of *Escherichia coli* from clinical samples.

Methods: A descriptive study was conducted at Microbiology Department of Modern Technical College, Sanepa, Lalitpur. A total of 300 *E. coli* isolated from different clinical specimens were included in the study for biofilm formation by Tissue Culture Plate (TCP) method and inhibition by EDTA solution. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller Hinton agar according to CLSI guidelines.

Results: Among the 300 *E. coli* isolates, 130 (43.3%) were found to be biofilm producers. Biofilm-producing *E. coli* showed significantly ($p < 0.05$) higher resistance to several antibiotics, including Amoxicillin (98.5%), Cefalexin (91.5%), Co-trimoxazole (87.7%), Cefixime (86.2%), Ceftriaxone (83.1%), Ciprofloxacin (73.8%), and Ofloxacin (73.8%). However, lesser drug resistance was observed among non-biofilm-producing *E. coli*. EDTA at concentrations of 5 mM and 10 mM greatly inhibited and reduced biofilm formation.

Conclusion: Among the isolated *E. coli*, 43.3 % were biofilm producer and those strains exhibited higher antimicrobial resistance compared to non-biofilm producers. EDTA was found to be effective in inhibiting biofilm formation. Hence, EDTA can act as antibiofilm agent to control and minimize biofilm asssociated bacterial infections.

Keywords: *Escherichia coli*, biofilm, EDTA, antimicrobial resistance.

INTRODUCTION

Escherichia coli is a Gram-negative bacterium which is a facultative anaerobic in nature. It is commonly found in the gut of humans and warm-blooded animals and external moist environments. Most strains of Escherichia coli are harmless but certain strains are responsible for different types of infections. Escherichia coli is known to be a most common bacteria responsible for a wide spectrum of clinical manifestations, and diseases including UTI, pneumonia, bacteremia, meningitis. *Escherichia coli* accounts for 70 to 95% of urinary tract infections (Parsek & Singh, 2003). Bacteria follow an ascending route of infection in 90% of urinary tract infections.

A biofilm is defined as a group of microbial cells attached to

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each other, and these cells are irreversibly attached to a substratum with the help of a self-produced matrix (Hall-Stoodley et al., 2004). Biofilms can form on both living and non-living surfaces (Ellis, 2010). The availability of key nutrients, chemotaxis towards a surface, bacterial motility, surface adhesins, and the presence of surface bacteria are some factors that influence biofilm formation. A large number of gram positive and gram negative bacterial species have the ability to form biofilms, such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter spp, Pseudomonas aeruginosa, Staphylococcus aureus,Staphylococcus epidermidis, Enterococcus spp, Acinetobacter spp* etc (Parsek & Singh, 2003). Biofilms are mainly associated with the tissue or indwelling medical devices such as catheters, implants leading to chronic, severe and persistent microbial infections.

Biofilms are currently estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial infections (Römling & Balsalobre, 2012). The bacteria enclosed within biofilms are extremely resistant to treatment due to insufficient drug concentration in certain areas of the biofilm. The bacteria located at the base of the biofilm are metabolically inactive and are thus resistant to certain antibiotics. They possess active antibiotic degradation mechanisms that help them avoid the accumulation of an effective drug concentration. Due to emerging antibiotic resistance, these bacteria are responsible for both nosocomial and community-acquired infections, leading to prolonged hospital stays, greater costs, and increased mortality. Multi-Drug Resistant (MDR) organisms can also spread to other healthcare settings and into the community (Kumar et al., 2013). Hospital-acquired infections by biofilm producer are associated with significant morbidity and mortality in today's healthcare environment (Taylor, 2004).

Various methods are used to detect biofilms in research, including Tissue Culture Plate (TCP), Tube Method (TM) , Congo Red Agar (CRA), bioluminnescent assay, piezoelectric sensor technology, and fluorescent microscopy examination (Christensen et al., 1982, 1985; Freeman et al., 1989). The TCP method is considered the gold standard (Freeman et al., 1989; Mathur et al., 2006; Panda et al., 2016).

Inhibition of biofilm formation can be achieved using EDTA solution, human serum albumin(HSA), ibuprofen, N-acetyl-L-cysteine (NAC), levofloxacin, and vitamin C. Biofilm formation by *Escherichia coli* can be prevented by coating medical devices with HSA alone or in combination with IBU or NAC. In addition, IBU and NAC could be useful in the treatment of urinary tract infections caused by *Escherichia coli* due to their inhibitory effect on both bacterial growth and biofilm formation (Naves et al., 2010). For the elimination of biofilms, various non-antibiotic drugs (NSAID, aspirin, EDTA, DMSO) and anti-biofilm agents (indole, imidazole, DNase I, alpha-amylase, garlic) are used. Multidrug-resistant (MDR) organisms are frequently implicated as the causative agents of acute and chronic infections contributing significantly to patient morbidity and mortality, as well as increased health care costs associated with treatment (Bergogne-Berezin et al., 1994; McGrath & Asmar, 2011). Since most of the multidrug resistant strains produce biofilm it seems necessary to provide continuous monitoring and determination of antibiotic susceptibility of clinical isolates (Babapour et al., 2016).

Therefore, this study was aimed to find biofilm production and to determine the antibiofilm activity of EDTA against *E. coli*. Antibiogram profiles and resistant pattern were also studied and compared with biofilm non producing *E. coli*.

METHODS

Study design and sampling

A Laboratory based cross-sectional study was performed at Microbiology Laboratory Department of Modern Technical College. The study was carried for 3 months from September 2019 to December 2019. The study included 300 *E. coli* isolates isolated from different clincal specimens in Department of Microbiology, Star Hospital, Nepal, using Standard Microbiology techniques of American Society of Microbiology. Escherichia coli was identified on the basis of morphological characteristics, Gram's Staining and Biochemical tests such as Triple Sugar Iron agar, Sulphur Indole Motiity agar, Urea agar, Citrate agar, Oxidase test, Catalase Test etc. The different clinical specimens were Urine, Pus, Sputum, blood, body fluids etc. Informed written consent was taken from the patients before studing their sample in the study in Star Hospital. *Escherichia coli* isolates were revived in microbiology laboratory of Modern Technical College by sub culturing on Blood agar (HiMedia, India) and Mac Conkey agar (HiMedia, India) and incubated at 37^0C for 24 hours.

Detection of Biofilm production

Tissue culture plate method (Singleton, 1997) was used for detection of biofilm production.Organisms isolated from fresh agar plates were inoculated in 10 ml trypticase soya broth with 1% glucose and incubated at 37OC for 24 hours. The cultures were then diluted 1:100 with fresh medium. Next 200 µl ofthe diuted culture of different stains were inoculated in each well of the sterile 96 wells flat bottom polystyrene tissue culture plates. The control organism was also incubated, diluted and added to tissue culture plate. Negative control well consisted of sterile broth. The plates were incubated at 37°C for 24 hours. After

incubation contents of each well were removed by gentle tapping. The wells were washed with 0.2 ml phosphate buffer saline (pH 7.2) four times. That removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using distilled water. Then solution of ethanol and acetone (80:20) mixture was kept on the well for uniform layer of biofilm producing organisms. Optical density of stained adherent biofilm was measured by using micro ELISA auto-reader at wavelength 570nm. The experiment was performed in triplicate and repeated three times. The average optical density(OD) values of each test strain and negative control were calculated, and the final OD values of a test strain were obtained by substracting the OD cutoff (ODc) value of the negative control from the average OD value of the test strain. The interpretation of biofilm production was done according to the criteria of Stepanovic et.al. (Stepanović et al., 2007). The ODC value had been specified as three standard deviations (SDs) above the negative control.

Interpretation of biofilm production

Table1: Interpretation criteria

Average OD value	Biofilm production
≤ ODc / ODc<~ ≤ 2x ODc	Non/ weak
2x ODc<~ ≤ 4x ODc	Moderate
> 4x OD	Strong

Optical density cut off value (ODc) = average OD of negative control + 3x of Standard deviation (SD) of negative control

Antibiotic Susceptibility Testing

Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (Watts JL 1999). Three to four isolated colonies of Escherichia coli were touched by straight wire and passed into peptone water. Mixed thoroughly. Incubated at 370C for 4 to 6 hours and turbidity compared to 0.5 MC Farland Standard. After then sterile swab was dipped into the broth and lawn culture was done on Muller Hinton Agar (MHA). Fifteen different commonly used antibiotics were tested in two different MHA plates. Following antibiotics were used: Amoxycillin (AMX 10 µg), Nitrofurantion (NIT 300 µg), Ofloxacin (OF 5 µg), Cefixime (CFM 5µg), Cotrimoxazole (COT 25 µg), Cephalexin (CN 30 µg), Amikacin (AK 30µg), Meropenem (MRP10 µg), Tigecycline (TGC 15 µg), Chloramphenicol (C 30 µg), Ciprofloxacin (CIP5 µg), Ceftriazone (CTR 30 µg), Amoxicillin/Clavulanic acid (AMC 30 µg), Cefpodoxime (CPD 10 µg), Tetracycline (TE 30 µg). The results were interpreted according to criteria set by CLSI. *E. coli* ATCC 25922 was tested as quality control.

2.3. Determination of MDR isolates

The isolates resistant to at least 3 antimicrobial agent classes was determined as MDR isolates (Magiorakos et al., 2012).

Prepatation of EDTA solutions

A 10mM EDTA solution was prepared by dissolving 3.72g of disodium EDTA in 1000 ml distilled water. Then the dilution of this solution was made in distilled water for the prepatation of 5mM of EDTA.

Inhibition of biofilm formation

The assay was done using the microtiter plate assay (SarojGolia et al., 2012) in a 96-well microtiter plate (Greiner Bio-one, Stuttgart, Germany), in the absence and presence of EDTA (5 and 10 mM), in triplicates. The optical density was measured at 570 nm with ELISA reader (BioTek®, MQX 200, USA) and the degree of biofilm formation was estimated (SarojGolia et al., 2012).

Statistical analysis

Data entry was performed using Microsoft Excel 2013, followed by analysis using SPSS verson 20 (IBM Corporation, Armonk, NY, USA).

Ethics statement

Ethical clearance was taken from Institutional Review Board (ERB) of Nepal Health Research Council (NHRC), Nepal (Ref No 1469).

RESULTS

Distributions of clinical specimens

During the study period, a total of the 300 *E. coli* isolates from different clinical specimens were subjected for observation of biofilm formation (Figure 1). Different clinical specimens included in this study are listed in table 2. These specimens were collected from 214 females and 86 males.

Table 2: Distribution of clinical specimens

Specimens	Number of *E. coli*	Percent
Urine	226	75.3
Pus	38	12.7
Sputum	17	5.7
Blood	9	3.0
Synovial fluid	7	2.3
Ascitic fluid	3	1.0
Total	**300**	**100**

Frequency of Biofilm producing organism

Out of 300 *E. coli*, 130 (43.3%) were found to be biofilm producer by Tissue Culture Plate method (Table 3).

Table 3: Showing frequency of biofilm production

Biofilm production	No. of isolates
Yes	130 (43.3%)
No	170 (56.7%)

Category of Biofilm production

Out of 130 *E. coli*, 41 isolates were found to be strong biofilm producer and 71 isolates as a moderate and 18 isolates as a weak biofilm producer (Table 4).

Table 4: Different category of biofilm formation

Biofilm Production	No. of isolates	Percent
Strong	41	31.5
Moderate	71	54.6
weak	18	13.9
Total	**130**	**100**

Distribution of MDR among biofilm producers and non-producers

High number of MDR phenotypes were found in both groups. Out of 130 biofilm producing *E. coli*, 119 (91.5%) isolates were MDR phenotypes and out of 170 biofilm negative *E. coli*, 87 (51.2%) isolates were MDR phenotypes (Table 5).

Association between biofilm producers and MDR

Biofilm producing organisms showed relatively high drug resistance against all the antibiotics tested as compared to non-biofilm producing organisms (Table 5).

Table 5: Biofilm formation and MDR

		Biofilm Producer		P-value
		Yes	No	
MDR	**Yes**	119	87	0.001
	No	11	83	

Chi-square test was applied and the p-value <0.05 was considered significant.

Antimicrobial resistance in Biofilm producing and non-producing *E. coli*

Biofilm producing *E. coli* were resistant to many antibiotis tested. Majority of the isolates were resistant to Amoxicillin (AMX 98.5%) followed by Cefalexin (CN 91.5%) then Co-Trimoxazole (COT 87.7%), Cefixime (CFM, 86.2%). High numbers were also found resistant to Ciprofloxacin (CIP 73.8%) and Ofloxacin (73.8%). Lesser numbers were resistant to Nitrofurantoin (NIT 16.9%), Amikacin (AK 20.8%) and Meropenem (MRP 6.9%). None of the isolates were found to be resistant to Tigecycline (Table 6).

High drug resistance was also observed among non-biofilm producing isolates but were less in percent as compared to biofilm producing isolates. Many isolates were found to be resistant to Amoxicillin (AMX 84.1%) followed by Cefalexin (CN 74.1%), then, Cefpodoxime (CPD 71.2%) and Cefixime (CFM 62.2 %). Many isolates were also found resistant to ciprofloxacin (CIP 62.9%) and ofloxacin (62.9 %). Less isolates were found resistant to Nitrofurantoin (NIT 8.8%), Amikacin (Ak 8.2%), Meropenem (MRP 2.4%). None of isolates were found resistant to Tigecycline (Table 7).

Inhibition effect on biofilm formation by EDTA

Five millimole of EDTA was added in microtiter plate and experiment conducted. There was reduction of biofilm formation by the organism after addition of EDTA solution. There was high decrease in Strong and moderate biofilm producer. Many isolates were observed to be weak and negative biofilm producer (Figure 2).

Next concentrations of 10 millimole of EDTA was used as biofilm inhibitor. It was observed there was very high reduction of biofilm formation by the organism after addition of 10millimole EDTA solution. It was found there was very high decrease in Strong and Moderate biofilm producer. Few isolates were found to be weak biofilm producer and many were non-producer (Figure 3).

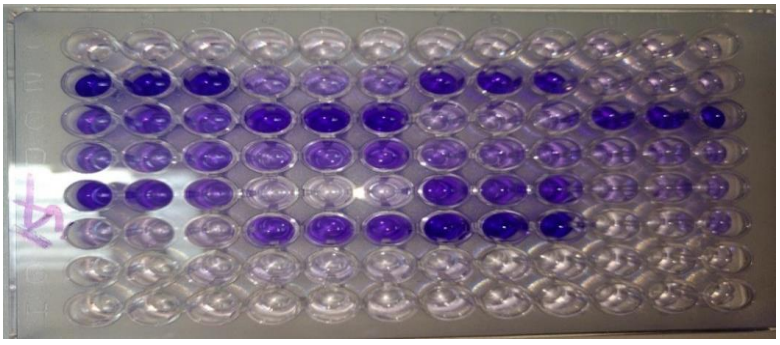

Figure 1: 96 well plate showing biofilm production (positive=purple)

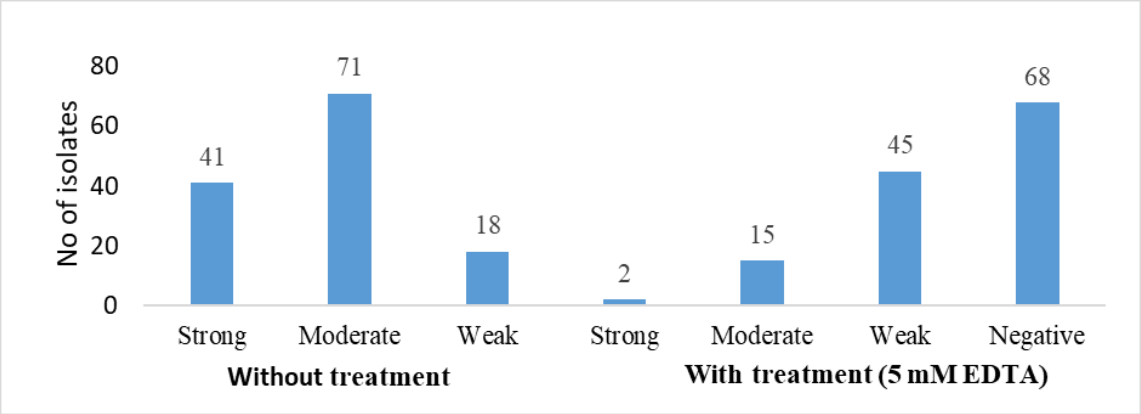

Figure 2: Inhibition effect of EDTA (5mM) on biofilm formation on strong, moderate and weak producers.

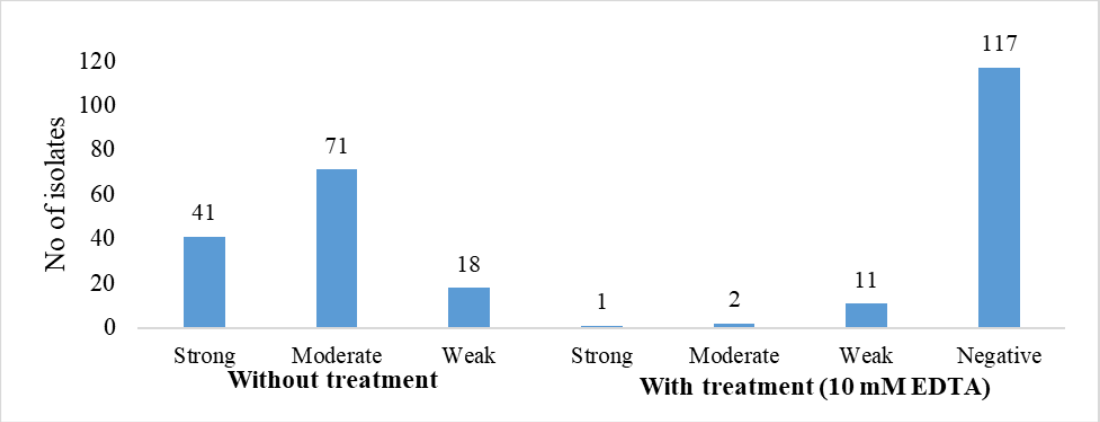

Figure 3: Inhibition effect of EDTA (10 mM) on biofilm formation on strong, moderate and weak producers.

Table 6: Antibiotic resistance pattern of Biofilm producing *E. coli* (n=130)

Antibiotics	Sensitive %	Resistance %	Intermediate %
NIT	57.7	16.9	25.4
AK	64.6	20.8	14.6
CIP	20.0	73.8	6.2
COT	11.5	87.7	0.8
CTR	9.2	83.1	7.7
AMX	0.0	98.5	1.5
MRP	81.5	6.9	11.5
AMC	16.2	61.5	22.3
CPD	3.1	81.5	15.4
C	74.6	10.8	14.6
TE	24.6	62.3	13.1
TGC	100.0	0.0	0.0
OF	20.0	73.8	6.2
CFM	10.8	86.2	3.1
CN	8.5	91.5	0.0

Table 7: Antibiotic resistance pattern of non-biofilm producing *E. coli* (n=170)

Antibiotics	Sensitive %	Resistance %	Intermediate %
NIT	72.9	8.8	18.2
AK	72.9	8.2	18.8
CIP	31.8	62.9	5.3
COT	40.6	57.6	1.8
CTR	27.1	62.9	10.0
AMX	11.2	84.1	4.7
MRP	90.6	2.4	7.1
AMC	28.8	45.9	25.3
CPD	17.1	71.2	11.8
C	84.1	10.6	5.3
TE	34.1	50.6	15.3
TGC	100.0	0.0	0.0
OF	31.8	62.9	5.3
CFM	27.6	68.8	3.5
CN	25.9	74.1	0.0

DISCUSSION

E. coli is the most frequent cause of urinary tract infections. Besides it is also responsible for several clinical manifestations such as bacteremia, septicemia, abscess, meningitis etc. It possess several virulence markers to produce disease and one of them is the biofilm formation (Jabalameli et al., 2012). Bacteria in biofilm display dramatically increased resistance to many antibiotics (Tayal et al., 2015) and confers resistance against host defence mechanism.Biofilm also leads to spread of antimicrobial resistance by favouring horizontal gene transfer of resistance and virulence factor causing generation of more and more virulent strains (Costerton et al., 2005). The ineffectiveness of antibiotics has lead to development of novel agents that can prevent, control or eliminate biofilm without involving the development of potentail resistance mechanism in biofilm associated infections (Alandejani et al., 2009).

Biofilm related infections are more troublesome and expensive to treat (Lebeaux et al., 2013). In this study, of 300 Escherichia coli isolates tested, 130(43.3%) isolates were found to be biofilm producer. However, in the study conducted by Shrestha et al., 2023, there was lesser prevalence of biofilm formation of 13.7 % and by Sanchez et al., 2013 study of 12.8% to be biofilm producer. There were many studies reporting high prevalence of biofilm production. In the study conducted by Dumaru et al., 2019 from Dharan Nepal, the prevalence of *Escherichia coli* forming biofilm were of 60.33% and from Kathmandu, Nepal by Dahal et al., 2023, reported 51.9 % biofilm producer and Chaudhary et al., 2019 reported 70% isolates as biofilm producers. In another study conducted by Shrestha et al., 2023, there was found very high prevalence of biofilm producing *E. coli* (97%).

Biofilm producer (130, 43.3%) were subdivided into Strong (41, 31.5 %), moderate (71, 54.6%) and weak or non-biofilm producers (18, 13.9%). This was similar to the study conducted by Dahal et al., 2023, in which out of 41 (51.9%), 8 (19.5%) were weak biofilm producer, 21 (51.2%) were Moderate biofilm producer and 12 (29.3%) were Strong biofilm producers. The study carried out by Hassan et al., 2011, the percentage of isolates forming strong or moderate biofilm were 71 (64.7%) and weak/non-biofilm were 39 (36.3%) among a total of 110 isolates. In study conducted by Elahe Tajbakhsh et al., 2016, the biofilm positive phenotype strains were classified as highly positive (6, 6 %),

moderate positive (80, 80%) and weakly positive (14, 14%).

In this study, biofilm producing organisms were found to be more MDR compared to non-biofilm producing organisms. Among 130 biofilm producing organisms, 119(91.5%) were MDR phenotypes and from 170 non-biofilm producing organisms 87 (51.2%) organisms were MDR. Association between Biofilm producing isolates and MDR were statistically significant (p-value= 0.001). In study conducted by Dahal et al., 2023, 54.3% biofilm producing isolates were MDR compared to 21.6% non biofilm producing isolates as MDR. There was very high percent of MDR reported by chaudhary et al., 2019, of 87.5% among biofilm producing *Escherichia coli* isolates. This higher antibiotic resistance among biofilm producers might be due to the close contact of organisms in biofilm, activity of the exopolysaccharide matrix, growth rate alteration, pH and osmotic variation and resistant gene or plasmid gene transfer among the isolates within a biofilm matrix (Shashikala et al.,2016).This high prevalence of MDR may be due to acqusition of various drug resistance mechanisms such as beta-lactamase enzymes, efflux pumps, decreased drug uptake besides biofilm formations (Tenover, F. C. 2006)(Parajuli et al., 2016).

The rates of antibiotic resistance of biofilm producing *Escherichia coli* were found to be high. The highest drug resistance was observed to amoxicillin (98.5%), followed by cefalexin (91.5 %), and Co-trimoxazole (87.7%). High resistant to Amoxycillin (92.2%) was also reported by Shrestha et al., 2023. Dahal et al., 2023, reported 85.7 % of amoxycillin resistance, and Chaudhary et al., 2019 reported 87.5% Amoxycillin resistance. Dahal et al, 2023 also reported as 66.1 of Ciprofolaxin resistance, 71.4 % of Ceftraxione resistance which was similar to this study. Biofilm non producers were also found to be MDR but relatively lesser than biofilm producing *Escherichia coli*. However, all the biofilm producing and non producing *Escherichia coli* isolates were Sensitive to Tegecycline. The results of the present and previous study show that high resistivity is shown by the biofilm producers than none producers, but it is not necessarily for each biofilm producers to be resistant to the antibiotics.

Biofilm producing *Escherichia coli* cause multiple prosthetic device mediated infections, leading to severe complications and ultimately resulting in high morbidity, mortality, medical costs, and hospital stay. Thus there is a critical need for identifying therapeutc strategies for biofilm formations and for effective treatment of biofilms (Jabra-Rizk et al., 2006). There are number of chemicals and methods that inhibit biofilm production by the pathogen. One of the method is incoporating EDTA solution. In this study we tested 2 different concentrations of EDTA solution against biofilm producing *Escherichia coli* isolates. The results revealed that using EDTA with concentrations 5mM and 10 mM inhibited biofilm production. A study conducted by Shrestha et al., 2023 in Nepal using different concentrations of EDTA of 0.5mM, 1mM, 2mM, 4mM, and 5mm among gram negative isolates, it was found 5mM was most effective. The Study conducted by Gawad et al., 2017 showed that EDTA with concentrations 10mM and 20mM inhibited biofilm formation by strong and moderate biofilm producing UPEC by 45.8 and 78.8% respectively.

This study has provided some information regarding biofilm formation, inhibition and antibiogram of *Escherichia coli* but this study be considered with some limitations. This study was conducted only for short duration. This study was based on phenotypic method of biofilm production as molecular methods and sophisticated microscopy techniques were constrained. Antibiotic susceptibility test was performed by disc diffusion method with commercially available antibiotic discs. No MIC and MBC were performed. Drug resistant genes were not identified. Thus further studies with a large sample size including drug resistant genes sholud be done to establish biofilm production and inhibition of *Escherichia coli* including other potential gram positive and gram negative isolates.

Conclusion

High percent (43.3%) of the *Escherichia coli* were found to be biofilm producer by Tissue Culture Plate techniques. Biofilm producing *Escherichia coli* were significantly ($p<0.05$) resistance to many antibiotics such

as Amoxicillin, Cefalexin, Co-Trimoxazole, Cefixime, Ceftriaxone, Ciprofloxacin, Ofloxacin as compared to biofilm non-producing *Escherichia coli*. There was also observance of more MDR among biofilm producer than among non biofilm producer. It was also noted that EDTA at a concentration of 5mM and 10mM greatly inhibited and reduced the biofilm formation.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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