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Molecular Detection of Antimicrobial Resistance Genes in *Biofilm* Forming *Escherichia Coli*

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ABSTRACT

Antibiotic resistance in *Escherichia coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine. This study was carried out to detect the location of antibiotic-resistant gene in biofilm forming *E. Coli* isolates by molecular approaches. The investigated genes included *CEF (E1)* Cefidinir and *AZT (E2)* Aztreonam. According to biochemical experiment, *E.coli* isolates were recognized from sewage water. After that Antibiotic susceptibilities of *E. coli* isolates were tested against 14 antimicrobial agents by the disk diffusion method**.** The resistance of *E. coli* was found against cefidinir and Aztreonam. Biofilm forming ability of *E. Coli* was investigated by Microtitre plate assay, cover slip assay and tube assay. *E. coli* shows biofilm forming ability by all 3 methods. Further, we have done molecular characterization of resistant *E. Coli*. Detection of the 16s rRNA gene using universal primers was done. PCR technique was used to detect specific antibiotic resistance genes. The results indicate that biofilm forming resistant isolate *EC1 (E. coli* strain 1) contains Cefidinir resistance gene at 742bp on 100bp ladder marker and *EC2 (E. coli* strain 2) contains Aztreonam resistance gene at 478bp in the context of 100bp ladder marker. Both biofilm forming resistance isolates of *E. coli* harbour antibiotic resistance genes. The present study explores; *in vitro* qualitative estimation of biofilm production of *E. Coli* isolated from sewage and correlates it with antibiotic resistance.

Keywords: *Escherichia coli*, Biofilm, antibiotic-resistance genes, Aztreonam, Cefdinir, PCR (Polymerase chain reaction)

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INTRODUCTION

The increase in antibiotic resistance is a threat to global health[1,2]. There is worldwide concern about the appearance and rise of bacterial resistance to commonly used antibiotics. In this regard, programs for monitoring resistance have been implemented in many countries for the purpose of protecting the health of humans as well as animals[3,4,5]. These programs usually monitor indicator bacteria such as *Escherichia coli*. In water, bacteria from different origins (human, animal, environmental) are able to mix and resistance evolves as a consequence of promiscuous exchange and shuffling of genes, genetic platforms, and genetic vectors[6]. Microbial indicators, in particular coliform bacteria and *Escherichia coli*, have been established for assessing the microbiological safety of drinking water. The presence of *E. Coli* in water is an indicator of fecal contamination and implies that pathogenic bacteria, viruses, and protozoa may also be present.

Escherichia coli is usually a commensal bacterium of humans and animals. Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia[7,8]. The treatment of illnesses caused by this bacterium often requires antimicrobial therapy. The decision to use antimicrobial therapy depends on the susceptibility of the microorganism and the pharmacokinetics of the drug for achieving the desired therapeutic concentration at the site of infection and thus clinical efficacy[9]. Surveillance data show that resistance in *E. Coli* is increasing for antimicrobial agents that have been in use the longest time in medical science. The past 2 decades have witnessed major increases in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporin's. For example, a study of the susceptibility of *E. Coli* isolates recovered from hospitals during a 12-year period (1971– 1982) shows no major change in resistance to any of the antimicrobial drugs tested.

E. Coli is the most prevalent infecting organism in the family of gram-negative bacteria. Some of the strain of *E. Coli* causes bloody diarrhea, anemia, UTI or kidney failure which can lead to death; most strain of *E. coli* produced a potent toxin – Shiga toxin that is harmful for the lining of the small intestine[10]. *E. coli* have strong biofilm forming ability. Biofilms are composed of microcolonies of bacterial cells that are distributed in a matrix which consists of exopolysaccharides, proteins, salts and cell material in an aqueous solution. The matrix takes about 85% of the volume of a biofilm. Bacterial biofilms are reported to be the most common cause of persistent inflammation[11,12]. *E coli* isolates shows positive association between virulence and biofilm. Now a days molecular techniques, especially polymerase chain reaction

(PCR), have been widely used to study antimicrobial resistance genes. Due to the excessive use of antibiotics in bacterial disease, the purpose of this study was to determine antimicrobial resistance gene in biofilm forming *E. Coli* isolates from sewage.

MATERIALS AND METHODS

Isolation and identification of *E. coli*

E. Coli strains were isolated from sewage samples. The sewage sample were inoculated on nutrient agar and MacConkey agar media plate and incubated 37°C for 24 hr. The colonies of isolated organism have been sub culture on nutrient agar plate and pure culture were obtained in various selective media such as EMB agar, SS agar medium. Green metallic sheen isolates were considered to be *E. Coli* and the presumptive colonies were biochemically tested for growth on triple sugar iron agar (TSI) and lysine iron agar (LIA), and for oxidative/fermentative degradation of glucose, citrate utilization, urease production, indol fermentation, tryptophan degradation, glucose degradation (methyl red test) and motility. The *E. Coli* isolates were stored in tryptic soy broth (Merck, Germany) with 15% glycerol at –20 °C [13]. Colony confirmation was performed using molecular methods (PCR). Molecular confirmation of clones was determined according to the 16S rRNA gene region from *E. Coli* described by Sabat et al [14].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084), as recommended by the National Committee for Clinical Laboratory Standards[15,16]. The antimicrobial agents tested and their corresponding concentrations were as follows: Norfloxacin(10 µg), Aztreonam (30 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Nalidixic acid (30 µg), Nitrofurantoin (300 µg), Cefuroxime (30 µg), Gentamycin (10 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Ceftazidime (30 µg), Cefixime (5 µg), Cefdinir (5 µg). A small inoculum of *E. coli* isolate was inoculated on Mueller-Hinton plates and antibiotic discs were placed on the plates, spacing them well to prevent the overlapping of inhibition zones. After incubating the inoculated plates aerobically at 37 °C for 18 to 24 h, the susceptibility and resistance of the *E. coli* isolates to each antimicrobial agent was measured and the results were interpreted in accordance with criteria provided by NCCLS[17]. *E. Coli* ATCC 25922 was used as quality control organisms in antimicrobial susceptibility determination. The isolated resistant strains of *E. Coli* were further purified.

Detection of biofilm formation

This study was carried out by three methods for detection of biofilm formation in *E. coli*. Microtitre plate assay (quantitative assay), Cover slip assay (qualitative assay) and Tube assay.

In Microtitre assay, *E. coli* strains (10^9 cfu/30µl) were cultured in BHI in 96-well microtitre plates at 37ºC for 48 h. After incubation broth was aspirated and wells were washed with PBS. 0.5% crystal violet stain was added for 5 min. The plates were then washed with tap water and 200 µl of 95% ethanol was added. The biofilm formation was considered positive when an optical density at 492 nm was equal or more than 0.2 **.**

In Coverslip assay, biofilms of *E. Coli* were grown as follows, Two sterile culture flask were filled with 50 ml of BHI broth and sterile 18 mm diameter glass microscope cover slip was added to each flask, and culture flask was plugged by sterile cotton. Each Flask was inoculated with defined volume of overnight culture. The Flask were incubated microaerobically at 37ºC for 48 h. Glass cover slips containing attached biofilm was removed from flask and rinsed briefly with PBS and stained with 0.5% crystal violet for 5 min. Stained biofilms were observed microscopically.

In Tube assay, biofilm of *E. Coli* grown on glass test tube and stained by 0.5% crystal violet for 5 min. Stained biofilms were observed visually in day light.

Molecular characterization *E. Coli*

DNA extraction

Two biofilm forming resistant strains of *E. Coli- CEF(E1), AZT(E2)* were selected for molecular characterization. Both strains were subcultured overnight in Luria-Bertani broth (Merck, Germany) and genomic DNA was extracted using a Hi Pura™ bacterial genomic DNA purification kit, according to the manufacturer's instructions. DNA electrophoresis was run in 1.8 % (w/v) agarose gels in a horizontal electrophoresis system (Genei, Banglore, India). Gels were stained with ethidium bromide and visualized under ultraviolet light in a LABNET TM-26 (Edison, NJ, USA) transiluminator.

Primers and PCR assay

16s rRNA gene detection achieved by PCR. Amplification of isolated DNA by 16s rDNA using the universal forward (F-5’-GAT TAG ATA CCC TGG TAG TCCAC-3’) and reverse primer (R-5’-CCC GGG AAC GTA TTC ACC G-3’) was done. The amplified DNA was observed in agarose gel electrophoresis.

Antimicrobial gene detection

The presence of genes associated with resistance to cefidinir *(CEF)* and Aztreonam *(AZT)* in *strain E1* and *E2* were determined by PCR and the set of primers used for each gene is shown in

Table 1.

PCR reactions were performed in a total volume of 25 µl, including 3mM MgCl2, 500mM KCl, 100mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µm of each dNTP (Fermentas), 1 µm primers, 2.5 IU of Taq DNA polymerase (Fermentas), and 5 µl (40–260 ng/µl) of DNA. Amplification reactions were carried out using a DNA thermo-cycler (Eppendrof Mastercycler, Eppendorf-Nethel-Hinz GmbH, Biorad, Germany) as follows: Three min at 95 °C, 26 cycles of denaturation carried out. Each consisting of 1 min at 94 °C, Annealing occurred at 55 °C for 1 min, followed by a final extension step of 10 min at 72 °C. Amplified samples were analyzed by electrophoresis in 1.8% agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp increments (100 bp DNA ladder, Fermentas) was used as a size standard.

Table 1 Primer sequences used for PCR identification

Primer Name	Primer Sequences
16S r RNA	F-5'-GAT TAG ATA CCC TGG TAG TCCAC-3'
	R-5'-CCC GGG AAC GTA TTC ACC G-3'
Aztreonam	F-TCGCCTGTGTATTATCTCCC
	R-CGCAGATAAATCACCACAAT
Cefdinir	F-TGGCCAGAACTGACAGGCAAA
	R- TTTCTCCTGAACGTGGCTGG

RESULTS AND DISCUSSION

In this study *E. Coli* strains were isolated from sewage and identified by morphological, cultural and biochemical characteristics.

Antimicrobial resistance

The results for the antimicrobial susceptibility testing of *E. Coli* strains against 14 antimicrobial agents are shown in Table 2. Data were interpretated as susceptible, intermediate or resistant by measuring zone of inhibition. The results showed that the isolated strain of *E. Coli* found resistant to Aztreonam (15mm ,zone of inhibition) and Cefdinir (0mm). Whereas, higher senstivity was noted to 5 antibiotics; Norfloxacin (17mm), Ceftriaxone (22mm), Gentamycin (15mm), Amikacin (20mm) and Ofloxacin (24mm) . *E.coli* strains found intermediate susceptibility to 7 antibiotics; Cefotaxime (22mm), Nalidixic acid (16mm), Nitrofurantoin (15mm), Cefuroxime (17mm), Ciprofloxacin (14mm), Ceftazidime (15mm), Cefixime (17mm). In this study we were found only 2 resistant strain of *E.coli* against Cefidinir and Aztreonam (fig.1), they were named as *CEF(E1)* and *AZT (E2)* respectively. The Enterobacteriaceae family has been linked to well known antibiotic resistant gene pools. These genes are transferred into the normal flora of humans and animals[18], where they exert a strong selective pressure for the emergence and spread of resistance in both pathogenic and commensal bacteria. Eventually they

find their way into the environment via wastewater, manure and sewage sludge[19,20]. Recreational water and wastewater have been identified as a potential source of resistant bacteria in the environment [21,22,23].

Table 2 showing Antibiotic susceptibility of *E. coli* isolated from sewage

Antibiotics	Zone of inhibition (mm)	Result
Norfloxacin (NF^{10})	17mm	Sensitive
Aztreonam (AT^{30})	15mm	Resistant
Cefotaxime (CX^{30})	20mm	Intermediate
Ceftriaxone (FR^{30})	22mm	Sensitive
Nalidixic acid (NA^{30})	16mm	Intermediate
Nitrofurantion (FU^{300})	15mm	Intermediate
Cefuroxime (CR^{30})	17mm	Intermediate
Gentamycin (GM^{10})	15mm	Sensitive
Amikacin (AK30)	20mm	Sensitive
Ciprofloxacin (CIP^{5})	14mm	Intermediate
Ofloxacin (OF^{5})	24mm	sensitive
Ceftazidime (CZ^{30})	15mm	Intermediate
Cefixime (FX^{5})	17mm	Intermediate
Cefdinir (CN^{5})	No Zone	Resistant

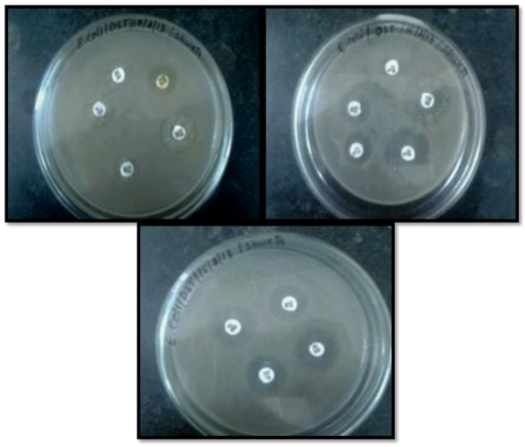

Figure. 1 Showing results of Disc diffusion test

Biofilm formation

In the present study, we observed that both resistant isolates *CEF(E1)* and *AZT(E2)* of *E. coli* shows ability to form biofilm on microtitre plate, glass coverslips and glass test tube. Readings of Optical Density (OD) of biofilm formation on Microtitre plate found to be 2.457 and 0.912 at 492 nm wavelength for *EC1* and *EC2* respectively. Both the values are more then 0.2, which indicates both the strains have strong biofilm forming ability. Figure 2 shows biofilm formation of *E. coli* on microtitre plate. Figure 3 shows the strong biofilm formation by resistant strain on coverslip. moreover tube assay results were also positive for both the resistant strains (fig. 4). *E. coli* are able to colonize the intestinal surface. Bacteria that form biofilms have been shown to

be highly resistant to antimicrobial therapy[24,25]. Adherence and production of a biofilm by *E.coli* on different biomaterials and medical devices has been documented[26]. The development of a biofilm is initiated when bacterials cells attached to surface and begin to excrete slimy, glue-like substances, which serve to anchor the cells[27]. The biofilm structure provides protection to the cells against host-defense mechanisms, phagocytosis, biocides, hydrodynamic shear forces and antibiotic treatment[28] . *E. coli* forms biofilms on abiotic surfaces in medical and industrial settings[29]. Bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infections. It has been estimated that 65% of microbial infections are associated with biofilms[30,31,32].

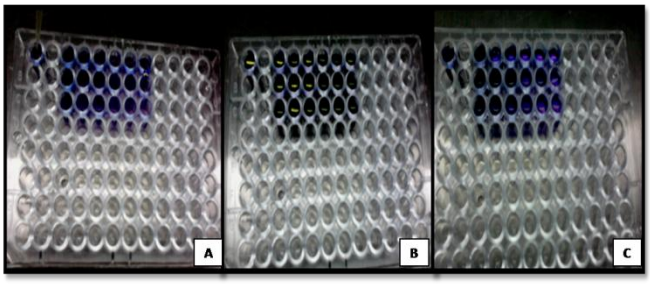

Figure 2 showing Microtiter Assay for *E. coli* biofilm

(A) Microtitre plate containing crystal violet for staining of biofilm.

(B) Front view of Microtitre plate containing biofilm.

(C) Microtitre plate containing 95% ethanol.

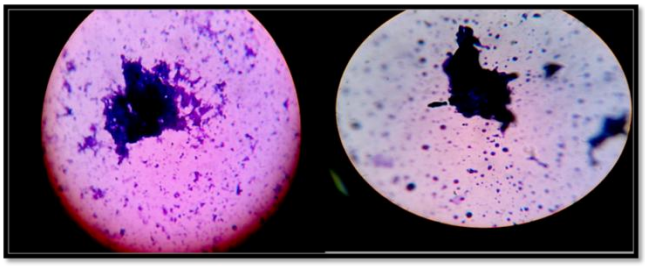

Figure 3 Microscopic view of Biofilm formation of *E. coli* by cover slip assay.

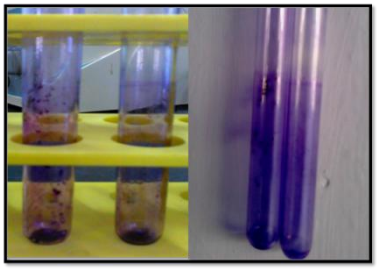

Figure 4 Biofilm formation of *E. coli* by Tube assay.

Molecular Characterization

DNA from resistant strains of *E. Coli* were purified using Hi Pura™ bacterial genomic DNA

purification kit and analysed on agarose gel electrophorosis. Figure 5 shows the total DNA of *EC1* and *EC2* strains found at 10000bp on 1kb ladder. 16s rRNA gene of *E. coli* were detected by PCR amplification of isolated DNA using 16s rRNA universal primer. Figure 6 shows the 16s rRNA gene of *EC1* and *EC2* strains found at 612bp on 100bp ladder marker.

Species specificity of *E. Coli* DNA was identified by 16s rRNA gene detection.

Antimicrobial resistant gene of *EC1* strain was detected by amplification of isolated DNA using Cefidinir; forward and reverse primer. Cefdinir resistant gene found at 742bp on 100bp ladder marker. The DNA of *EC2* strain was amplified with Aztreonam; forward and reverse primer by using PCR technique. Aztreonam resistant gene found at 478bp on 100 bp ladder marker (fig.7).

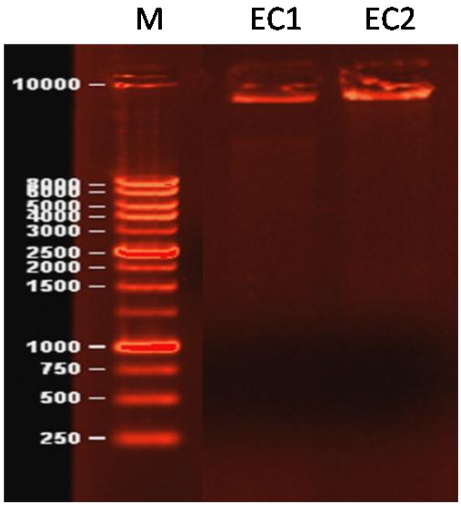

Figure 5 *E. coli* total DNA on 1.8% agarose gel

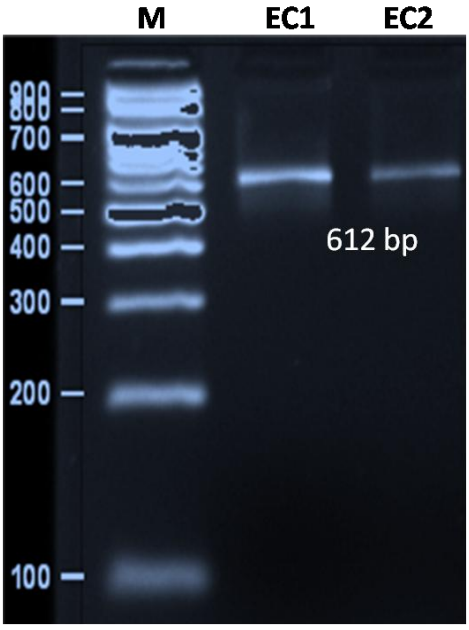

Figure 6 showing 16S rRNA gene found at 612 bp on 1.8% agarose gel after PCR

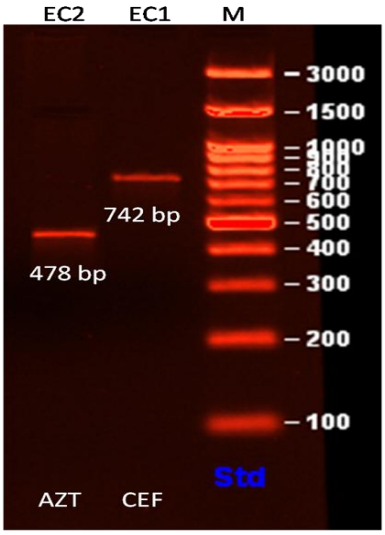

Figure 7 Gel electrophoresis of the PCR products of Antibiotic resistance gene.

M- Molecular weight marker 100bp ladder

EC1- CEF resistant gene found at 742 bp

EC2- AZT resistant gene found at 478 bp

CONCLUSION

We observed that strong biofilm forming ability of E. coli isolates indicate strong association with pathogenicity. In our study, we have isolated *E. coli* from sewage water and identified by biochemical characterization. Antibiotic susceptibility of isolated E. *coli* against 14 antimicrobial agents was checked by disk diffusion method. Two resistant strain *CEF(E1)* and *AZT(E2)* were obtained. Both resistant strains shows strong biofilm forming ability. Biofilms are marker for pathogenicity in *E. coli.* The pathogenic potential of *E. Coli* strains is thought to be dependent on the presence of virulence factors (VFs)[33,34]. Molecular approaches are very sensitive and reliable methods for the identification of microorganism. we have also detected the 16s rRNA gene in biofilm forming resistant *E. coli* using universal primers. Antibiotic resistance gene against aztreonam and cefedinir also detected using specific primers by the help of molecular techniques**.**Thus our results suggests that antimicrobial resistance is more prevalent in biofilm forming *E. coli.*

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