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Isolation and identification of Biofilm-producing *E. coli* from drinking water.

Aiman Fatima[1,2], Zulfiqar Ali Mirani[2], Anila Siddiqui[2], Shaista Urooj[1,4] & Tanveer Abbas[1]

[1]Department of Microbiology, University of Karachi, Karachi-Pakistan.
[2]Microbiological Analytic Center, PCSIR Laboratory Complex Karachi Center, Karachi-Pakistan.
[3]Department of Microbiology, Federal Urdu University of Arts, Science, and Technology, Karachi-Pakistan.
[4]Department of Life Sciences, Shaheed Zulfikar Ali Bhutto Institute of Science and Technology, Karachi-Pakistan.

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Corresponding Author Email: syedaaf@hotmail.com
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Abstract

Background: The increasing rate of water-borne diseases in Karachi demands the characterization of associated pathogens. Nowadays, water-borne infections become more resistant to treatment due to biofilm formation and excessive use of antibiotics. Biofilm formation in water deteriorates the quality of water. This study identified and characterized biofilm-producing E. coli in drinking water.

Methodology: Water samples were analyzed for heterotrophic plate count and total coliforms, fecal coliforms, and E. coli. The Kirby disk bar diffusion method was used to investigate antibiotic susceptibility in biofilm-producing E. coli. The Congo red and tube ring methods were used to identify biofilm producers. The effect of biofilm formation on the hydrophobicity of E. coli was performed by the BATH method. The soft agar method determines the colony spreading ability of biofilm and non-biofilm producers. Molecular characterization of virulence genes of E. coli was performed by a PCR. Scanning electron microscopy for biofilm construction was conducted.

Results: The total 120 water samples were tested for heterotrophic plate count and total coliforms, fecal coliforms, and E. coli. 78% were unfit in this study, and 21.66% were fit. 38 E. coli strains were found in water samples. According to findings, the hydrophobicity of biofilm-producing isolates increased with the incubation period. Colony-forming unit drops one logarithm in the biofilm state compared to the planktonic stage. Biofilm producers were more resistant to antibiotics. The virulence genes, pet, lt, and stx2, were used for the molecular characterization.

Conclusion: The presence of biofilm-producing E. coli in drinking water is alarming, and it indicates inappropriate treatment of the water supply system. To prevent rapid water-borne diseases, adequate actions are required to control drinking-water biofilm producers.

Keywords

Biofilm, Hydrophobicity, Antibiotic Resistance, *E. coli*.

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Introduction

The existence of life on earth mainly depends on the water. Water is the basic need of life. Food and water are the main integral source of nutrition for the body[1]. It provides a medium for metabolism in the human body. Water is also known as "aqua" and "Adam's ale"[2]. Water comprises 75% of body weight in infants and 55% in the elderly. Loss of 1% water from the body will compensate within 24 hours to keep the body water in balance. The human body requires safe and healthy water (potable water) for survival. Water quality is determined by physical, chemical, and biological parameters. Biological traits include the bacteriological characterization of water. This research is concerned with fecal indicators and biofilm-producing bacteria in drinking water.

In nature, microbes favor living in a community compared to living in a planktonic style[3]. Biofilm is a survival strategy for microbes to compete for space, nutrients, and stress[4]. Biofilm formation is a dynamic and complex process where microbes of the same species or different species adhere to the surface. Biofilm formation has a great impact on public health and wealth[5]. This process is mainly composed of 5 stages, reversible attachment, irreversible attachment, EPS production, maturation of biofilm, and dispersion of biofilms[6]. Bacterial species adhere to the surfaces by different appendages. The extracellular matrix of biofilm appears as slimy – sticky like glue, which becomes denser with the period. In biofilm consortia, bacterial species communicate with each other by releasing chemical signals known as quorum sensing. After biofilm maturation, exhaustion of nutrients starts, which leads to the dispersion of biofilms.

In the health care system, hospital-acquired infections occurred mostly due to the biofilm development in medical equipment. It can deteriorate medical devices and water and food reservoirs and supply systems. Biofilm production in drinking water leads to many water-borne diseases such as respiratory, gastrointestinal, skin-related infections, and neurological disorders. These infections are very difficult to treat due to biofilm formation. Biofilm development plays a major role in increasing antibiotic resistance, especially in hospital-acquired infections. In drinking water, biofilm-producing *E. coli* is the most studied pathogen, and the WHO and the US EPA have approved it as a fecal indicator bacterium in drinking water[7]. According to WHO guidelines, total coliform, fecal coliform, and *E. coli* should be zero in 100ml of drinking water for human consumption.

Methodology

Analysis of water samples

The most probable method (MPN) was used to assess 120 water samples (ISO 9308-2:1990) for determining the following parameters: total coliforms, fecal coliforms, and *E. coli*.

- Heterotrophic Plate Count:

This method determines the total number of live heterotrophic bacteria in a water sample. The pour plate method was used to count heterotrophic plates[8].

- Confirmation Test: Oxidase Test and IMVIC Test:

Both tests were carried out similarly to Mirani et al study[9].

- Isolation of Escherichia Coli from Unfit Water Samples:

Water samples were evaluated for coliform, total coliform, and fecal coliform (*E. coli*)[10].

Antibiotic resistance pattern

The Kirby bar disk diffusion method determined the antibiotic resistance profile of *E. coli*[11].

Phenotyping characterization of slime producing bacteria

- Biofilm Detection by Congo Red Method

The congo red method was used to first detect biofilm growth. In this procedure, brain heart infusion agar was made by mixing 50 grams of sucrose per liter with 0.8 grams of Congo red dye per liter. This mixture is put into sterile empty plates and set aside to solidify. At 37°C, plates were incubated aerobically for 24 to 48 hours. Black colonies with a dry crystalline appearance indicated

positive outcomes, whereas non-biofilm producers remained pink in color[12].

- Tube Ring Method

The Tube ring method was also used to verify the results of the congo red method. In this method, isolates from water samples were inoculated in 10 ml of trypticase soy broth with 1% glucose. Incubate these broths for 24 hours at 37°C. Cultures were decanted after 24 hours, and tubes were washed with phosphate buffer saline (Ph 7.3). After drying, the tubes were stained with 0.1 crystal violet, and Deionized water was used to remove the excess discoloration. Tubes were dried inverted, and the growth of a biofilm ring was observed. A noticeable line runs along the tube's wall and bottom, indicating a positive biofilm producer. The development of rings at the liquid interface was not regarded as a sign of biofilm formation.

Quantification of biofilms

Subjective isolates were inoculated in glass tubes with 1 ml of sterile TSB medium overnight. These tubes were incubated at 37 degrees for 24 to 48 hours in a standing position. The supernatant was discarded. The tubes were washed three times with distilled water to eliminate undesired cells and debris. The tubes were patted dry using a paper towel. The tubes were stained with 3 percent crystal violet and left to sit at room temperature for 30 minutes to stain adherent material. Remove the stain and rinse the tubes three times with distilled water. To release the confined molecules of crystal violet from the biofilm, 1 ml of 70% ethanol was poured into these tubes. To record the absorbances at 563 nm wavelength, transfer 100 ul from the tubes into a 96-well microtiter plate[13,14].

- Enumeration of Biofilm Population

Enumeration of the biofilm population was performed in the following manner. Subjective isolates were grown for 24 to 48 hours at 37°C in flasks containing BHI broth and 4mm glass slides. Glass slides were washed with phosphate buffer saline to eliminate undesired debris after biofilm formation. 3 ml of this PBS was placed into sterile 5 ml tubes, and the tubes were vortex for 2 minutes at 3000 rpm to separate the cells from the biofilms. After vortexing, the bacteria are counted using the agar dilution plate technique. Each sample containing dislodged bacteria was serially diluted ten times. To calculate the accurate count of the biofilm population, a 10 ml inoculum was seeded[15,16].

Bacterial hydrophobicity method by bath method.

Hydrophobicity of biofilm producers was determined by the BATH method as described by Butina et al & Carniello et al[17,18].

Evaluation of colony variants and detecting of persister cells

The biofilm from the glass slides was resuspended in regular saline (1 ml). To disrupt the cell clusters, homogenize the mixture for 30 seconds. This sample is serially diluted and plated on Baird-Parker agar, Cetrimide agar, and EMB agar, depending on the selective medium used. Subculturing well-isolated colonies on tryptone soya broth and incubation at 37°C for 24 hours revealed the stability of colony variant cells. This phase is carried out six times more[19].

- Test for the characterization of small colony variants

Test for the characterization of small colony variant can be performed as suggested by Mirani et al[9].

Colony spreading of *E. coli*

Colony spreading of *E. coli* was performed as described in[20,21]. Isolates were grown in Luria-Bertani broth for 24 hours. The needle was used to locate colonies in the middle of the soft agar plate. Incubate the plates for 24 hours, 32 hours, and 48 hours at 37°C. Examine the plates for colony shape and colony size measurements. This experiment is repeated three times.

Effect of temperature on biofilm producers

The dip method was used to observe the effect of temperature on biofilm producers. The Nutrient broth was inoculated with positive isolates and incubated at 37°C for 24 hours. The full loop

inoculum was transferred from nutritional broth to 50 ml TSB bottles containing glass slides. For 48 hours, these bottles were incubated at 25°C, 35°C, and 45°C. Glass slides were rinsed with phosphate buffer saline after 48 hours (PBS). These slides were dyed with crystal violet for 1 minute before rinsing five times with distilled water. After drying, ethanol was used to fix the slides. At 589 nm, observed for the optical density of these tubes. This experiment is repeated three times.

Scanning electron microscopy

Scanning electron microscopy was performed in a flask containing a 4 mm glass slide, subjective isolates were cultured in BHI broth for 24 hours. Glass slides were washed with distilled water after incubation to eliminate any undesired material. For 30 seconds, slides were stained negatively with 0.2 percent uranyl acetate. GOEL-JEM-1200 EX II electron microscope was used to investigate these slides[22].

Polymerase chain reaction

Primers for identifying *E. coli* virulence genes were brought in from corporations[23,24].

PCR calculations

The entire reaction mix for Pcr amplification of extracted DNA is 25 µl. Then, as directed in the supplement, use a specified primers. 12.5 µl of Taq Master Mix, 0.5 µl of each reverse and forward primer, nine µl of sterile MilliQ water, and 2.5 µl of Genomic DNA lysate were used for PCR amplification. Depending on the primer/gene type, the DNA was amplified using a thermal cycler (BIO-RAD, iCycler) at varied annealing temperatures. An agarose gel containing ethidium bromide (EtBr) and TBE (Tris-borate-EDTA) buffer was used to visualize the PCR amplified products. A 100bp DNA ladder (Bioron®) was put into 10-15l µl of amplified product. After that, electrophoresis was performed at 100 volts for 40–60 minutes. The agarose gel was examined under a UV-trans-illuminator lamp, and a photograph of the gel was taken with a Nikon® digital camera.

Statistical analysis

Data analysis was performed by using SPSS version 17.0 for frequencies. Data present in the form of tables and figures were the mean values of 3 independent experiments.

Results

A total of 120 water samples were evaluated in this study based on heterotrophic plate count and the presence of total fecal coliforms and *E. coli*. According to WHO Guidelines for Drinking Water Quality, 4th Edition. Only 26 water samples fit human consumption, and 94 were unfit water samples (Table 1).

Table 1: Water analysis of drinking water (n=120).

Variables		N(%)
Fit Samples		26(21.66)
Unfit Samples		94(78.2)
Analysis Method	MPN method (Detection of coliforms, fecal coliforms, & total coliforms)	59(62.76)
	Heterotrophic plate count method	35(37.23)
Isolations from unfit samples	*E. coli*	39(41.48)
	Other Isolates	55(58.51)

Antibiotic resistance pattern of *E. coli*

The Kirby bar disk diffusion method was used to determine the antibiotic susceptibility of isolates. Antibiotic resistance was higher in biofilm-forming isolates after a total of 16 antibiotics were employed (Table 3).

Interpretation of the zone of inhibition was matched by the Clinical and Laboratory Standards Institute CLSI (2015 and 2012) and The European Committee on Antimicrobial Susceptibility Testing EUCAST (2016).

Biofilm screening

The ability of isolates to create extracellular material was tested via biofilm screening. For biofilm screening, two techniques were employed phenotypically. The Congo red method and the tube ring method are both effective. Biofilm-positive isolates form a black color colony in the congo red method, whereas non-biofilm producers create a colorless colony. Biofilm producers develop a ring along the tube line using the tube ring method.

Result of quantitative assessment

Biofilm was examined quantitatively and qualitatively. It was discovered that *E. coli* isolates to form biofilm at a mature stage of 48 hours. At 578 nm, biofilm absorbance was measured at 24 and 48 hours, indicating that biofilm matures after 48 hours instead of 24 hours (Table 2).

Table 2: Characteristics of monospecies biofilm-producing *E. coli*.

S.no.	Optical density		Enumeration of biofilm & non-biofilm population		Hydrophobicity		Small colony variant or persister cells
	OD at 24 hrs.	OD at 48 hrs.	PBP at 24 hrs.	BP at 48 hrs.	PBS at 24 hrs.	BS at 48 hrs.	
1	0.13	0.55	$1*10^5$	$1*10^3$	0.57	0.46	<1cfu
2	0.15	0.59	$1*10^6$	$1*10^3$	0.58	0.45	<1cfu
3	0.15	0.57	$1*10^6$	$1*10^3$	0.53	0.46	<1cfu
4	0.17	0.59	$1*10^6$	$1*10^4$	0.54	0.53	<1cfu
5	0.34	1.13	$1*10^6$	$1*10^4$	0.90	0.62	$1*10^1$
6	0.33	0.98	$1*10^6$	$1*10^3$	0.85	0.61	$1*10^1$
7	0.37	0.97	$1*10^5$	$1*10^3$	0.83	0.56	<1cfu
8	0.46	0.51	$1*10^5$	$1*10^4$	0.62	0.52	<1cfu
9	0.37	0.73	$1*10^6$	$1*10^4$	0.77	0.51	$1*10^2$
10	0.45	0.98	$1*10^5$	$1*10^4$	0.86	0.47	$1*10^2$
11	0.44	0.63	$1*10^6$	$1*10^4$	0.64	0.43	<1cfu
12	0.49	1.07	$1*10^5$	$1*10^3$	0.81	0.62	<1cfu
13	0.41	0.52	$1*10^6$	$1*10^4$	0.72	0.63	<1cfu
14	0.41	0.53	$1*10^6$	$1*10^4$	0.73	0.64	$1*10^1$
15	0.47	0.55	$1*10^6$	$1*10^4$	0.71	0.66	$1*10^2$
16	0.45	1.04	$1*10^5$	$1*10^3$	0.91	0.64	$1*10^2$
17	0.49	1.04	$1*10^5$	$1*10^3$	0.88	0.65	<1cfu

OD-Optical Density; PBP-Pre-Biofilm Population; BP-Biofilm Population PBS-Pre-Biofilm Stage; BS-Biofilm Stage

Enumeration of biofilm population

Biofilm enumeration was done at two stages: pre-biofilm and post-biofilm. It was used to identify the isolated population before and after biofilm development. According to the findings, the population was higher at the pre-biofilm stage than at the biofilm stage. It means that individual cells are more numerous at the pre-biofilm stage. The number of individual cells was reduced at the biofilm stage, as illustrated in (table 2).

Bacterial hydrophobicity method by bath method

The hydrophobicity of biofilm-producing isolates determines the ability of microorganisms to attach themselves to hydrophobic materials. According to the findings, all 17 *E. coli* biofilm-producing isolates show a rise in hydrophobicity as the incubation period increases. (Table 2).

Evaluation of colony variants and detecting of persister cells

Biofilm development necessitates numerous metabolic changes in microbes. Some metabolically inactive cells were found in the old biofilm, which were not present in the pre-biofilm stage. Biochemical tests were used to characterize Persister cells. According to the findings, *E. coli* develop a limited amount of SVSc or Persister cells (Table 2).

Table 3: Antibiotic resistance pattern of biofilm and non-biofilm-producing *E. coli.*

S. no.	Antibiotics	Biofilm producers (n=30)	Non-biofilm producers (n=50)
1	Clarithromycin (CLR) 15 ug	13(43)*	20(40)*
2	Doxycycline (DO) 30 ug	11(37)*	10(20)*
3	Ampicillin (AMP) 10 ug	15(50)*	20(40)*
4	Aztreonam (ATM) 30 ug	7(23)*$	10(20)*
5	Cefotaxime (FOX) 30 ug	10(33)*	12(24)*
6	Gentamycin (CN) 10 ug	14(47)*	10(20)*
7	Tetracycline (TE) 30 ug	22(73)*+	15(30)*
8	Exatapenem (ETP) 10 ug	10(33)*	5(10)*
9	Carbenicillin (CAR) 100 ug	20(67)*	12(24)*
10	Imipenem (IPM) 10 ug	10(33)*	12(24)*
11	Tigecycline (TGC) 15 ug	22(73)*+	15(30)*
12	Ciprofloxacin (CIP) 5 ug	15(50)*	10(20)*
13	Levofloxacin (LEV) 5 ug	10(33)*	8(16)*
14	Chloramphenicol (C) 30 ug	17(57)*	10(20)*
15	Erythromycin (E) 30 ug	15(50)*	10(20)*
16	Amoxycillin (AML) 30 ug	12(40)*	13(26)*

*No. Of Resistant Isolates; +Maximum Resistance in Biofilm Producers; $Minimum Resistance in Biofilm Producers

Effect of temperature on biofilm producers

The temperature affects biofilm-producing isolates. The influence of temperature was studied using 17 biofilm-producing *E. coli*. The biofilm production increases with the increase in temperature, and Biofilm formation increased at 35°C and 45°C compared to 25°C (Table 2).

Colony spreading of *E. coli*

Biofilm-forming isolates accounted for 17 of the 40 *E. coli* isolates, while non-biofilm-forming isolates accounted for 23. Comparatively, non-biofilm-forming *E. coli* biofilm-forming isolates do not affect the colony spreading of *E. coli* (Table 2).

Furthermore, colony spreading of biofilm-forming isolates was slowed in a small percentage of cases. The results show that colonies of biofilm-producing isolates had a higher cell mass density than non-biofilm isolates, which have smaller cells.

Scanning electron microscopy

The biofilm matrix formation in bacteria was studied using scanning electron microscopy. *E. coli* produces a tiny, dispersed biofilm. In the extracellular material of *E. coli*, discontinuous cells were observed.

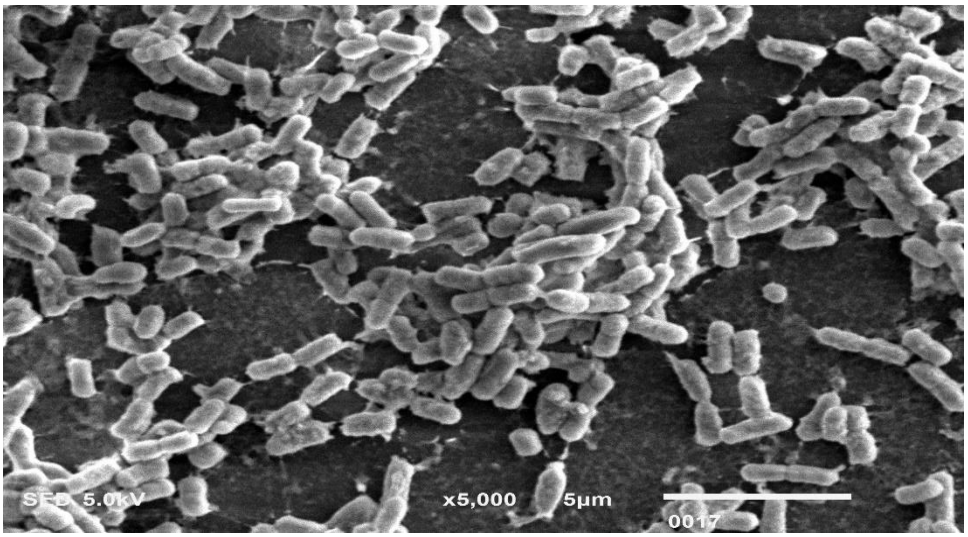

Figure 1: Biofilm producing *E. coli*.

Polymerase chain reaction

Several virulence genes were used to characterize *E. coli* at the molecular level. A total of ten virulence genes were used; however, only three of them, PET, AST A, and STX 2, were found in *E. coli* isolates.

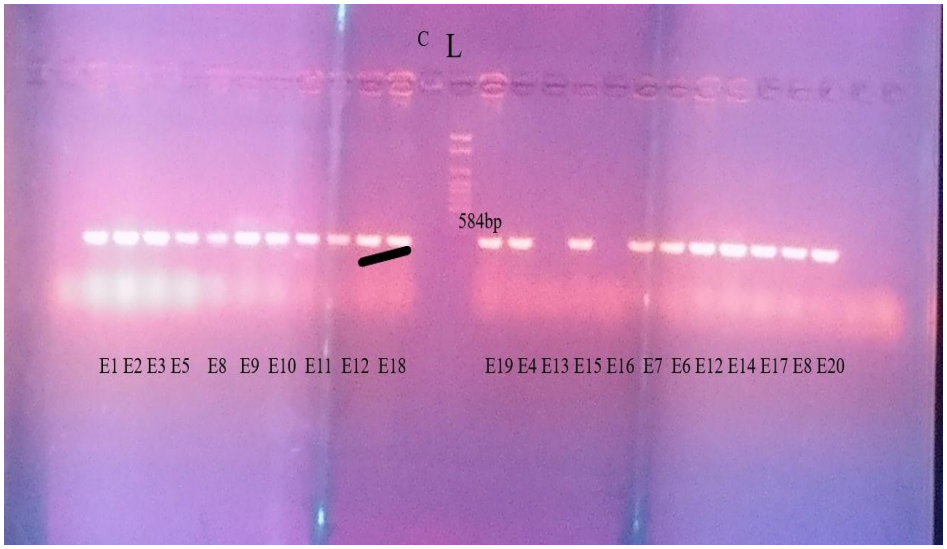

Figure 2: A STX 2 amplification of *E. coli* strains

L= DNA marker of 584 bp, C= negative control

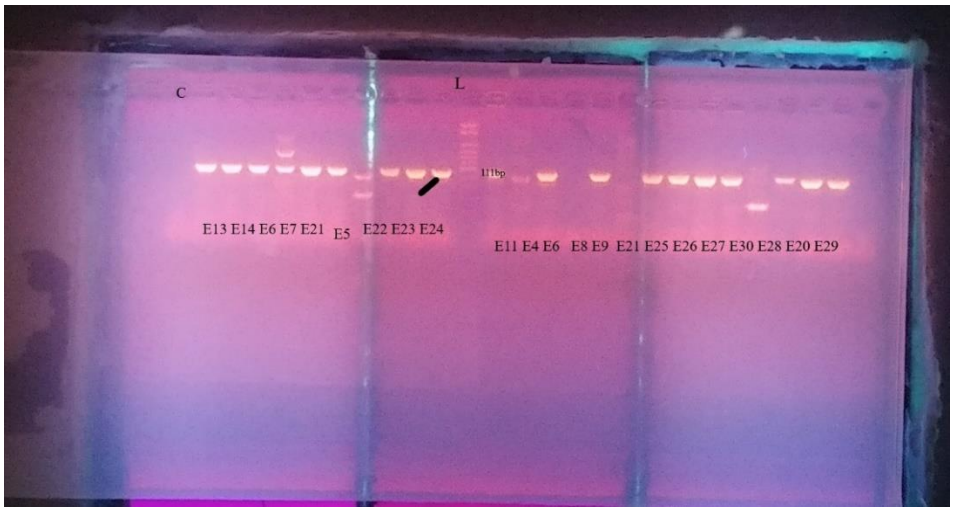

Figure 3: AST A amplification of *E. coli* strains
L= DNA marker of 111 bp and C= negative control

Discussion

Accessibility to safe drinking water is a basic need of human beings. It has been observed that fecal contamination in drinking water leads to many water-borne diseases. Water-borne ailments have been related to poor hygiene practices, which cause gastrointestinal infections in children[25]. Poor sanitation of water supply systems results in contamination of household surfaces[26]. This study collected water samples from diverse sources such as domestic water, industrial water, and water used in hospitals and pharmaceuticals. The results obtained from this study were alarming (Table 1) that is 78% of the water was contaminated, out of which 32% were contaminated with *E. coli*. In a recent study, *E. coli* is used as a fecal contaminant. A study conducted by Nowicki et al. found that *E. coli* is the widely used fecal indicator bacteria[27]. Research has developed new techniques to detect fecal contamination in drinking water, especially molecular points leading to new insights in this piece of research. There is an intense need to develop a surveillance system to detect bacterial pathogens. According to the WHO guideline (1998), three parameters for safe drinking water were standardized, including heterotrophic plate count, coliforms, and fecal coliforms.

Different chemical treatments in the water supply system increased antibiotic resistance and biofilm formation in *E. coli*. In this research Twenty-one, antimicrobial drugs were used. Antimicrobial drug resistance was divided into two parts comprising biofilm producers and non-producers. High resistance was observed in biofilm producers compared to non-biofilm producers (Table 3). Biofilm-producing *E. coli* showed the highest resistance to the tetracycline group of antibiotics. Group of monobactam antibiotics aztreonam) showed the highest activity towards biofilm producers. Rubab and Oh[28] concluded that Shiga toxin-producing *E. coli* (STEC) exhibited the highest resistance to erythromycin; Lin et al.[29] suggested that biofilm-producing genes in *E. coli* are responsible for higher resistance against antibiotic drugs; they observed that strains that carry pgaABCD and hipAB exhibited higher resistance. Current findings suggest that high antibiotic resistance in biofilm-producing *E. coli* leads to many bacterial water-borne diseases. A similar study conducted by Mahmud et al.[30] in Bangladesh revealed that drinking water collected from different sources was a major cause of water-borne diseases due to MDR, ESBL – producing strains of *E. coli* in large numbers.

Many methods can make identification of Biofilm producers. In this study, biofilm screening of isolates was performed using the congo red method and tube ring method. It has been

observed that biofilm formation by *E. coli* takes place in the later stages of the cell cycle (Table 2). After 48 hours of incubation, the optical density of biofilm producers was increased compared to non-biofilm producers. In this study, the population of *E. coli* at the planktonic stage was higher than the population at the biofilm stage. (Table 2). *E. coli* in pre biofilm stage overcome other species, but after biofilm formation, species like S. aureus and P. aeruginosa dominate *E. coli*. Similar results were observed in a study performed by Kuili Fang[31] that probiotic *E. coli* inhibits the biofilm formation of *E. coli* (0157). Probiotic *E. coli* that contains the Deg P gene can inhibit biofilm formation. Johnston et al.[32] observed that aryl polyenes (APEs) from Biosynthetic gene clusters allow the bacteria to interact with the surrounding environment, thus increasing the ability to form biofilm. The Biofilm formation by *E. coli* is affected by different environmental factors like pH, temperature, and stress. We observed the effect of temperature on biofilm. The favorable temperature for biofilm formation was 25 C and 35 C. This phenomenon was supported by Ma et al.[33], which grows Shiga toxin-producing *E. coli* at different temperatures. It was observed that hydrophobicity of biofilm producers *E. coli* increases with an increase in incubation period. (Table 2). This study was comparable with the study conducted by Klemen Bohinc et al.[34], in which they collected the uropathogenic strains of *E. coli* from catheters and grew them on multi-layer coated hydrophobic surfaces. They found a higher amount of biofilm formation on hydrophobic surfaces. De-la-Pinta et al.[35] found a direct relationship between hydrophobicity and biofilm formation. In the current study, molecular characterizing of *E. coli* isolates was performed for different virulence genes like Lt, Pet, astA, stx2, stx1, eacA, and Hyla. *E. coli* strains were positive for Lt, Pet, astA, and stx2. Awad et al.[36] identifies these genes in *E. coli* feces of cattle and buffalo calves. Gao et al.[37] isolated virulence genes from patients suffering from diarrhea Zil-e-Huma et al.[38] concluded that children less than two years of age were significantly affected by pathogenic strains of *E. coli*, which were positive for stx2 genes. Biofilm formed by *E. coli* is well organized and consists of small cells. This study concluded that the drinking water samples were contaminated with biofilm-producing fecal indicators. These samples were from different areas of Karachi but not from the whole city. In order to analyze the quality of drinking water in Karachi, we have to collect samples from every area, but this is very difficult because many illegal water supply systems are running in the city. Water quality is also dependent upon hygiene practices, but in our areas, very poor and unhygienic quality of life is present.

Conclusion

It was observed from the present study that water provided for human consumption in Karachi was highly contaminated with biofilm-producing *E. coli*, which were more resistant to the clinical antibiotic Poor hygiene practices results in water contamination. These contaminated water samples were the main source of diarrheal diseases. These diarrheal infections are increasing day by day, and their treatment becomes more difficult due to biofilm formation. Biofilm formation ability can be used for therapeutic purposes in a multispecies biofilm.

Conflicts of Interest

All authors confirm that they have no conflicts of interest.

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