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A Biofilm Formation Analysis of Environmental Escherichia coli Isolates

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Abstract

Microbial aggregates known as biofilms are kept alive as a community by the release of an extracellular matrix made up of proteins, DNA, and exopolysaccharides. Biofilm formation is a type of self-defence mechanism that microorganisms use to avoid stresses from the environment when they are in unfavourable environmental conditions. Long-term survival on both biotic and abiotic surfaces is made possible by these communities' exceptional resistance to disinfectants and antimicrobials. Being able to produce biofilm, E. coli a very versatile microbe that can survive in a variety of environmental conditions. In this study E. coli were isolated and identified from environmental samples using standard microbiological procedure. Total 420 environmental E. coli isolates were subjected to determination of biofilm formation by screening Congo Red Agar (CRA) method, all screened 315 biofilm forming E. coli isolates were subjected to qualitative Tube Method (TM) and quantitative Micro Titre Plate (MTP) assay. In this study from 315 screened E. coli isolates, 98% positive by TM method and 98% positive by MTP method. Biofilms lead to recurring infections, the failure of antibiotics, also industrial issues such as equipment contamination that reduces productivity. Therefore, research into biofilm-producing microorganisms is crucial.

Keywords: Biofilm, Environment, Escherichia coli (E. coli), Congo Red Agar Method (CRA), Test Tube Method (TM), Microtiter Plate (MTP) Method

1. Introduction

The ubiquitous multicellular life form of bacteria is called a biofilm. The bacteria embed themselves in an extracellular matrix (ECM), which provides protection by binding toxic substances and preventing predation by environmental microorganisms or immune cells [1].

Bacterial biofilms have emerged as a significant contributor to global health issues because of the host's immune defence system, antibiotic resistance, and other external factors. Biofilms are frequently observed in industry, food processing facilities, natural settings, and on the surface of medical equipment and bodily tissue [2].

E. coli is a remarkable adaptable microorganism, survive in a wide range of environmental settings. Faecal microorganisms have the potential to endure prolonged periods in soils, manure and water and therefore, they serve as an accessible source of contamination. Additionally, it may survive in wastewater under a variety of circumstances and frequently acts as an indicator of faecal pollution [3].

Pathogenic and commensal strains of E. coli can contaminate a variety of external habitats, such as fresh produce, drinking water, soil, and food processing facilities. The production of biofilms gives E. coli a general strategy to ensure persistence in a variety of conditions after sanitization treatments [1].

In food-producing facilities, the biofilm may serve as a reservoir for cross contamination of the products and as an environment for the dissemination of virulence genes and emergence of new pathogenic E. coli isolates. 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.

The adhesion of biofilms to surfaces becomes particularly significant when E. coli interacts with plants. When bacteria from soil, manure, or irrigation water attach themselves to plant tissues, they can form biofilms that eventually spread to a new mammalian host when the plant is consumed. Many recent enteropathogenic E. coli epidemics are caused by the consumption of contaminated fresh vegetables [4].

Hence, the aims of this study were to determine the presence of the most potentially virulent environmental E. coli isolates and to analyse their biofilm-forming ability using diverse methods.

2. Materials and Methods

In this study, 420 consecutive non duplicate E. coli were detected from various environmental sources of Surat City. The isolated E. coli was analysed for their biofilm production ability. Determination of biofilm formation was carried out using three methods 1) Screening method-Congo Red Agar (CRA) method, 2) Qualitative- Tube Method (TM) and Quantitative method- Microtiter Plate method (MTP).

2.1 Sample collection

Various environmental samples were collected from multiple sources to analyse the biofilm-forming ability of E. coli isolates. All samples were aseptically collected in sterile containers, which were clearly labelled with relevant metadata including the location, source type, time, and date of collection. To preserve microbial integrity, samples were immediately transported to the microbiology laboratory in insulated containers with ice packs, maintaining a temperature of approximately 4°C.

The collected environmental samples were categorized based on potential for human accessibility to E. coli and origin types of the sample into the following groups:

1) Direct human contact- Food (plant and animal sources), Water (Potable water)

2) Indirect human contact- Sewage, Animal Excreta, Surface, Soil & Manure.

2.1.1 Direct human contact

In accordance with the Indian Council of Medical Research's Standard Operating Procedures, ICMR Foodborne Pathogen Survey and Research Network, 2024, food and water samples from direct human contact were collected, processed and subjected to bacteriological examination [5].

2.1.1.1 Food sample: These samples are typed based on the origins.

Plant origins: Samples obtained from vegetables including carrots, cucumber, coriander leaves, cabbage, cauli flower, spinach, potatoes, green onion, bottle gourd, brinjal, fenugreek, green garlic, and ginger and fruits including apple, banana, chikkoo, mango, papaya, and watermelon were selected at random from Surat's local market. To avoid cross-contamination, collected sample units of each fruit and vegetable weighing between 100 g were gathered and put in different sterile plastic bags.

Animal origins: Samples from animal sources comprised milk, fish, and meat (chicken and goat) that were gathered from the Surat city local market. A sterile knife was used to cut a portion of approximately 100 g of beef and 250 g of fish from the top centre and other areas of the sample. The pieces were then gathered and placed in a wide-mouth jar. The milk samples from cow and buffalo farms were collected using a sterile utensil after being shaken or mixed, and around 20 mL was collected into a sterile container.

2.1.1.2 Water sample: 1–2 L of water samples were collected from various sources within Surat city, including lakes, wells, rivers, and tap water. The samples were collected in heat-sterilized, narrow-mouthed glass bottles pre-treated with freshly prepared sodium thiosulfate solution (1.8% w/v, 1.0 mL per Liter of water) to eliminate any chlorine residues. Before sampling, water was allowed to flow for 2–3 min. Samples were then taken 30 cm below the surface, with the bottle opening facing the direction of flow.

2.1.2 Indirect human contact

Samples from indirect human contact, such as sewage, surface, animal excreta, soil, and manure, were gathered using the procedures described by Cooley et al. (2013) and Lupindu (2017) [6, 7].

2.1.2.1 Sewage sample: Non-potable water samples, including water used for animals, birds, and irrigation were collected from various regions of Surat city. Sewage water samples were collected from the inlet pipes of various sewage treatment plants across Surat city. Approximately 20 mL of each water sample was collected in a sterile container.

2.1.2.2 Animal excreta sample: Fresh fecal samples from the upper surface of mammalian droppings were collected using transport swabs containing Cary-Blair medium (HiMedia, MS202), taking care to avoid cross-contamination from ground-borne microbes.

2.1.2.3 Surface sample: Samples were taken from surfaces such tables, meat vendors' hands and knives, various work areas in microbiological laboratories, toilet seats, and toilet door handles. A wet swab of transport media was used to obtain the surface sample. A Z-pattern of wet swabs was applied to the sampling surface. The swab was rubbed from left to right across the pre-marked surface area while maintaining a flat surface and applying even pressure. Wiped again from bottom left to top right and top to bottom. Swipe from top left to bottom right several times. After that, the swab was placed in sterile transport media.

2.1.2.4 Soil and Manure sample: Soil samples were collected from a depth of 2–5 cm below the surface. Approximately 100 g of each soil and manure sample were obtained in sterile plastic bags from various agricultural sites across Surat city.

2.2 Sample processing

For analysis of water and food samples, MC Broth was utilized as a preliminary (presumptive) test to detect coliform bacteria, especially E. coli. It also serves as an enrichment medium to increase the number of coliforms before confirmatory tests.

2.2.1 Direct Human contact

2.2.1.1 Food sample: From 100 g sample unit of each fruit and vegetables, aseptically weighed 25 g was mixed with 225 mL Phosphate Buffered Saline (PBS) (HiMedia, M1452) then swirled with a sterile glass rod for 20 min to thoroughly mix to homogenised sample. From 100 g meat sample, 25 g crushed into fine pieces using a sterilized scissor and homogenised in 225 mL of peptone water (HiMedia, M028) through mixing. From each fruit, vegetables and meat homogenates, 1 mL of the homogenate were inoculated into 9 mL of E. coli selective enrichment media MacConkey broth (MC broth) (HiMedia, M007). Then incubated at 37°C for 18-24 h for enrichment.

2.2.1.2 Water sample: From each potable water sample units 1 mL was inoculated into 9 mL MC broth and incubated at 37°C for 18-24 h.

2.2.2 Indirect Human contact

2.2.2.1 Sewage sample: From each sewage sample units 1 mL was inoculated into 9 mL MC broth and incubated at 37°C for 18-24 h.

2.2.2.2 Animal excreta sample: Animal excreta sample swab collected in transport Cary-Blair medium was transfer to 10 mL of MC broth and incubated at 37°C for 18-24 h.

2.2.2.3 Surface sample: After swabbing on the surface swab was inserted in the 10 mL test tube that contains sterile MC broth for enrichment and then incubated for 18-24 h at 37°C.

2.2.2.4 Soil and Manure sample: From the collected 100 g sample unit 25 g of each sample (Soil or Manure) were added to 225 mL of MC broth and mixed thoroughly by sterile glass rod then 1 mL of homogenate of each sample was added into 9 mL of MC broth and incubated at 37°C for 18-24 h.

Lactose fermentation leads to a colour change (e.g., purple to yellow), possibly with gas production, indicating a presumptive positive for coliforms/E. coli. Samples that MC Broth test preliminary positive for E. coli should undergo for completed tests, specifically to isolate and confirm the presence of E. coli.

2.3 Isolation and Identification of E. coli

Presumptive positive for E. coli MC broth cultures from the samples were plated on MacConkey Agar (HiMedia M081) for selective and differential isolation of E. coli.

Following incubation, MacConkey agar plates were examined to identify E. coli. A well-isolated, rose-pink lactose-fermenting characteristics colony on MacConkey agar was suspected of being E. coli were subjected to standard bacteriological examinations like Gram staining, motility testing, and growth characteristics on highly selective medium Eosin Methylene Blue (EMB) agar (HiMedia, M317), as well as standard biochemical tests for E. coli identification the IMViC reactions (I-Indole production test, M-Methyl red test, Vi-Voges-proskauer test, C-Citrate utilisation test), Urease production, and carbohydrates fermentation tests [8]. Finally, the isolates were phenotypically confirmed E. coli isolates.

2.4 Detection of microbial biofilm formation (BF): The E. coli isolates were then investigated for biofilm formation by screening Congo Red Agar (CRA) method, qualitative Tube Method (TM) and quantitative Micro Titre Plate (MTP) assay.

2.4.1 BF formation screening - CRA (Congo Red Agar) Method

For screening of biofilm forming E. coli isolates, CRA method was used which described by Yadav et al. (2018) [9]. CRA plates were prepared by adding 3.7 g of Brain Heart Infusion broth (BHI) (HiMedia, M210), 5% sucrose (Finar, 57-50-1) and 1 g of Standard Agar No. 1 (M1210) in 100 mL of distilled water. A concentrated aqueous solution of Congo red stain (8 g/L) (Finar, 0506SG005) was made and autoclaved at 121°C for 15 min apart from the other medium ingredients. The CRA plate was inoculated with 100 µL of the E. coli isolate suspension of bacterial density 0.5 McFarland turbidity standard (1.5 x 10^8 CFU/mL) using the streak plate method and plates were incubated at 37°C for 24 - 48 h. After incubation colour of colonies were observed for biofilm formation. Black colonies with a dry, crystalline quality were indicate biofilm formation, dark colonies with no dry crystalline morphology were interpreted as intermediate/moderate biofilm forming, while strains that did not form biofilms produced the red or white colonies [10].

2.4.2 BF formation qualitative detection- Tube Method (TM)

The qualitative Tube Method (TM), as described by Panda et al. (2016), was employed in this study to detect biofilm-producing microorganisms as a result of the occurrence of visible film [11]. The experiment was carried out three times. Briefly, A loopful (100 µL) of test organisms E. coli culture made from overnight culture plate, adjusted to 0.5 McFarland standard was inoculated to 10 mL of BHI broth with 2% sucrose in a sterile test tube. Every test tube was incubated at 37°C for 24 h. After decanting, the tubes were cleaned with PBS (pH 7.2) and allowed to dry. After that, it was stained for 30 min with crystal violet (0.1%) (HiMedia, TC510). Excess stain was washed with deionized water. Tubes were dried in an inverted position at room temperature the tubes were checked for the development of biofilm. Biofilm formation was demonstrated by a visible film around the tube's wall and bottom (Figure 1). Following tube examination, the results were noted as E. coli isolates shows biofilm production or lacking of biofilm production. The level of biofilm formation was categorized as semi quantitatively either strong, moderate considered Positive and Weak/absent as Negative.

2.4.3 BF formation quantitative detection-Micro Titre Plate (MTP) Assay

The Microtiter Plate (MTP) assay, a quantitative method for determining biofilm production, was performed as previously described briefly test, suspension of E. coli isolates were made from overnight incubating MacConkey agar plates, equivalent to 0.5 McFarland standard was inoculated in 5 mL BHI broth supplemented with 2% sucrose and incubated at 37°C for 18–24 h [12, 13]. Then it was diluted (1:100) with the same broth. Total of 200 µL of the broth culture was dispensed into wells of a sterile 96-well flat-bottom polystyrene microtiter plate (HiMedia, EP6) and broth without E. coli culture (media only) was used as a (blank/negative) control (c) to ensure that the medium was sterile and did not bind non-specifically. The plate was incubated at 37°C for 24 h without agitation to allow biofilm formation. After incubation, the wells were gently washed four times with phosphate-buffered saline (PBS, pH 7.2) to remove planktonic cells. The adherent biofilm was fixed for 1 h at 60°C, and then stained with 1% (w/v) crystal violet for 30 min and air-dried. After that excess stain was thoroughly cleaned with deionized water. Plates were kept at room temperature for completely dry. Then 200 µl of Glacial acetic acid solution (33%) was added to each well for re-solubilization of the dye bounded to the bacterial cells in the biofilm

layer, after 15 min the absorbance was measured using a microplate reader (Tulip Lisaquant-TS, Microplate Elisa Reader). All experiments were conducted in triplicate to ensure reproducibility.

The optical densities (OD) of stained adherent bacteria were measured at 570 nm. These OD values were interpreted as indicators of the bacteria's ability to adhere to surfaces and form biofilms. Based on the mean OD readings, isolates were categorized as non-biofilm producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer that shown in table 1. Where ODc represents the cut-off OD value defined as the mean OD of the negative control plus three standard deviations (SD). This classification allowed for a standardized, quantitative assessment of biofilm formation capacity.

Table 1: Classification of Bacterial Adherence by MTP Method

Mean Corrected OD Values for Biofilm Production Classification	**Calculated Mean OD Values**	**Biofilm Production**
$OD \leq ODc$	0.05	None
$ODc < OD \leq 2 \times ODc$	<0.120	Weak
$2 \times ODc < OD \leq 4 \times ODc$	0.120-0.240	Moderate
$OD > 4 \times ODc$	≥0.240	High

3. Results

3.1. Identification of E. coli Isolates

A total of 420 E. coli isolates were identified using standard microbiological procedures and confirmed as E. coli. Of these, 208 isolates were obtained from samples involving direct human contact—namely food (of plant and animal origin) and water. The remaining 212 isolates were obtained from samples involving indirect human contact, including sewage, excreta, surfaces, soil, and manure.

3.2 Biofilm Formation Analysis of the E. coli Isolates

3.2.1 Detection of microbial biofilm formation: Total 420 E. coli isolates were subjected to detection of microbial biofilm formation by numerous methods. In this study the results of biofilm formation of the E. coli isolates were analysed by using different screening, qualitative (Semi-quantitative) and quantitative methods as depicted in figure 1. Which gives schematic representation of the screening approaches used to evaluate biofilm formation in E. coli isolates. The methods included:

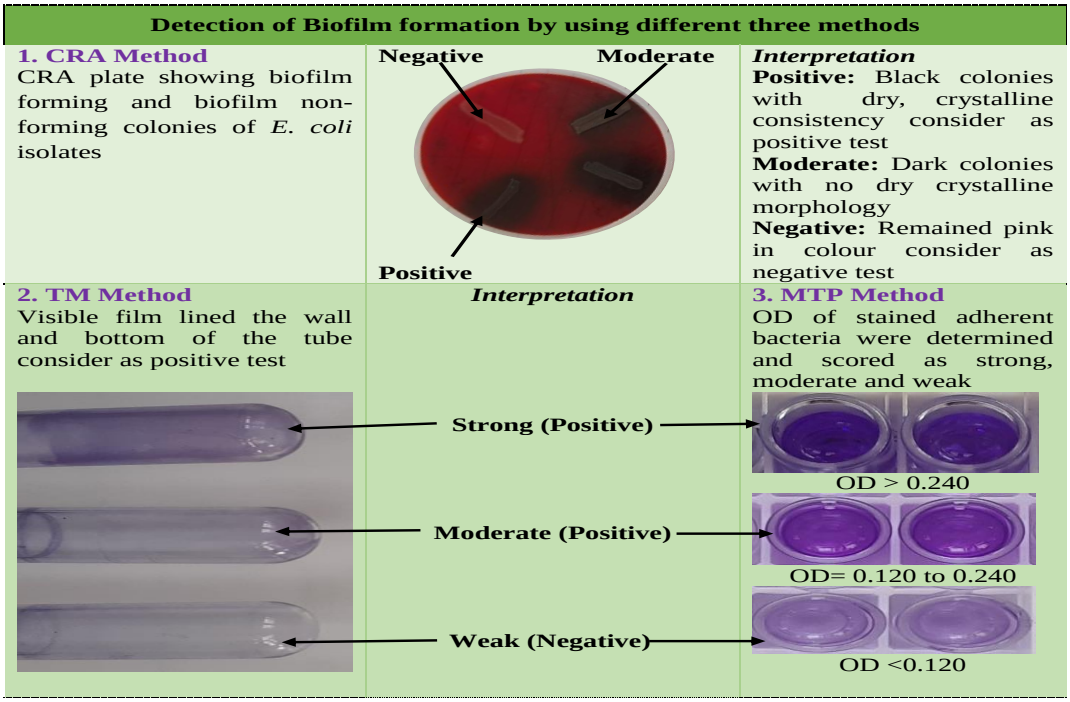
(A) Congo Red Agar (CRA) method — a qualitative assay based on colony morphology to indicate exopolysaccharide production.

(B) Tube method (TM) — a semi-quantitative visual assessment of biofilm formation on the walls and bottom of glass tubes after crystal violet staining.

(C) Microtiter Plate (MTP) assay — a quantitative colorimetric method based on optical density (OD_{570}) measurements of stained biofilms in 96-well plates.

Results from all three methods were compared to assess the consistency and reliability of biofilm detection in the environmental E. coli isolates.

Figure 1: Overview of Biofilm Detection Methods Used in This Study



3.2.2 Results of BF screening (CRA) Method:

Out of 420 isolates, 273 E. coli isolates show black colonies with a dry, crystalline quality were biofilm positive (BFP) isolates, 42 E. coli isolates show dark colonies with no dry crystalline morphology were interpreted as moderately biofilm positive (BFP), while 105 E. coli isolates that produced red colonies, considered as biofilm negative isolates (BFN). From screening total consider BFP E. coli isolates (n=315), 152 were from direct human contact sample and 163 were from indirect human contact. According to the CRA method, 75% of the environmental E. coli isolates screened biofilm-forming. In a study conducted by Chaitanya et al. (2021) reported that 58% of E. coli isolates from meat and water sample were positive for biofilm production by the CRA method [14].

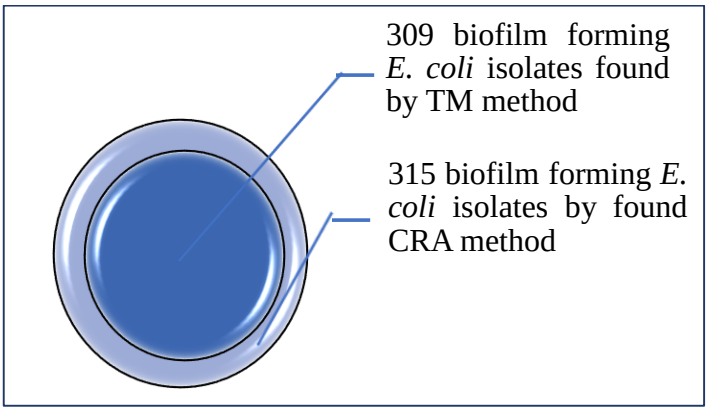
A previous study reported low accuracy of the CRA method for detecting biofilm formation [15]. Accordingly, in the present study, E. coli isolates classified as positive or moderately positive by the CRA method were further evaluated using the qualitative TM method and the quantitative MTP method.

3.2.3 Results of BF qualitative (semi-quantitative TM) method:

Of the 315 E. coli isolates subjected to the qualitative TM method, 309 were identified as biofilm-forming. Among these, 152 isolates originated from direct human contact samples, while 157 were from indirect human contact samples. 98% of the E. coli isolates were found to form biofilms, as determined by the tube method. According to different study by Kowalska et al. (2020) and Nouri Gharajalar (2017) the tube method revealed that 100% and 60% of the E. coli isolates from food samples were biofilm forming respectively [16, 17].

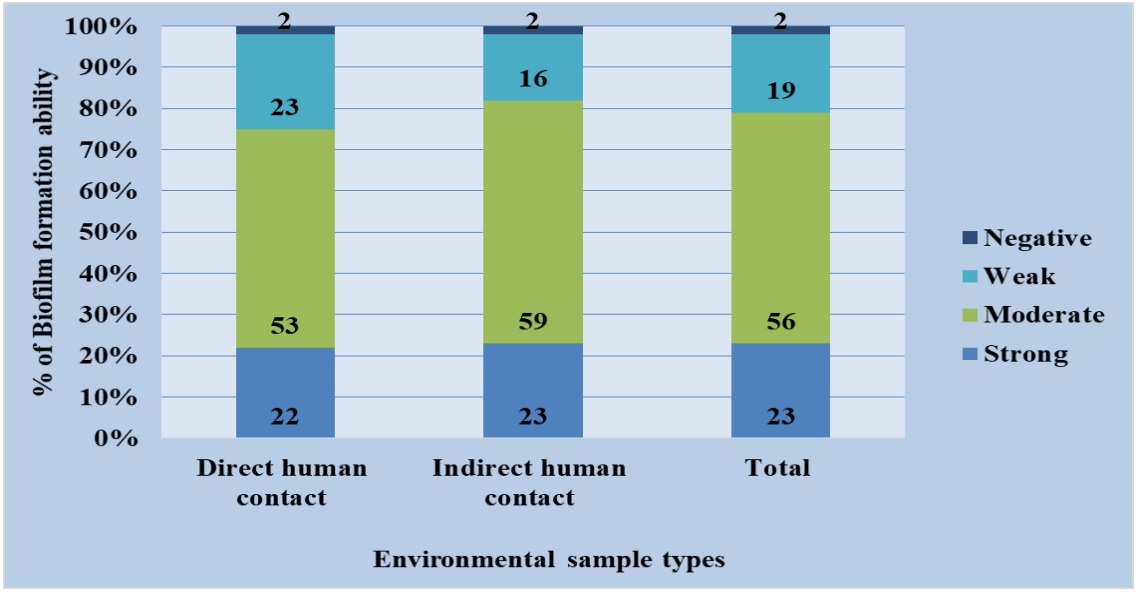
In this study, of the total 420 E. coli isolates, 315 were identified as biofilm-forming using the CRA method. Of these, 309 were further confirmed as biofilm-forming by the TM method, as illustrated in figure 2.

Figure 2: Biofilm Formation of E. coli Isolates by Using CRA and TM Method



3.2.4 Result of BF Quantitative (MTP method) Evaluation: Initially, all 315 E. coli isolates were screened for biofilm production using the CRA and the TM method. Following these preliminary assessments, the isolates were further evaluated quantitatively for the biofilm-forming ability using the MTP assay. This method allowed for the classification of biofilm formation into strong, moderate, and weak categories, based on the optical density (OD) readings obtained with an ELISA reader. A total of 309 E. coli isolates out of 315 were identified as biofilm-forming using the MTP method. Among these, 71 isolates exhibited strong biofilm formation—33 originated from direct human contact sources and 38 from indirect human contact sources. The 177 isolates showed moderate biofilm-forming ability, with 82 from direct human contact and 95 from indirect human contact. And remaining 61 isolates showed weak biofilm forming, among these 36 from direct human contact and 95 from indirect human contact. The percentage distribution of biofilm formation among E. coli isolates from different sample sources is illustrated in figure 3.

Figure 3: Percentage of Biofilm Forming Ability of E. coli Isolates from Diverse Sample Types



4. Discussion

E. coli is a diverse species of bacteria that, under certain conditions, can produce biofilms. We found a high prevalence of biofilm-forming E. coli in environmental samples. This ability has significant implications for both environmental survival and pathogenicity as well as contributes to antimicrobial resistance. E. coli remains a significant pathogen globally, with recent epidemiological data highlighting concerning trends, particularly in antimicrobial resistance (AMR). In our study, the presence of biofilm-forming environmental E. coli particularly typed in human contact sources suggests that such environment may serve as a potential reservoir for pathogenic bacteria, which could have significant implications for public health.

Abd El-Razek et al. (2023) found that 80% of E. coli isolates tested positive for biofilm development using the MTP method, with 26% of them producing strong biofilms, 34% producing intermediate biofilms, and 20% producing weak biofilms from drinking water [18].

According to study of Barilli et al. (2020) 6.5% strains of E. coli isolated from meat sample were found to be strong biofilm producer, 10.9% strains were moderate biofilm producer and 82.6% strains were weak biofilm producer by MTP method [19].

Several studies have reported a significant proportion of environmental E. coli isolates capable of forming biofilms. Abd et al. (2020) reported that all tested E. coli isolated from environmental samples were capable of forming biofilms by MTP method as strong (41%), moderate (34.7%) or weak (8%) [20].

In the study by Rega et al. (2022) using the MTP method, 5.5% of E. coli isolated from meat samples were classified as strong biofilm producers, 13.2% as moderate, and 31.9% as weak or negligible producers [21].

Our findings are consistent with these reports, showing a high prevalence of biofilm-forming E. coli in sources with frequent human contact. Environmental samples are susceptible to E. coli contamination, according to this study, and the majority of the isolates had the ability to form biofilm. Here we are concluded that from the human contact environment total 420 E. coli were isolated, among them 315 E. coli isolates were biofilm forming by CRA method, 309 by TM method and 309 by MTP method. The formation of microbial biofilm is a kind of self-protection behaviour of microorganisms in unfavourable environmental conditions, which helps them in avoiding environmental stresses. Therefore, the E. coli biofilm has become a hidden danger of microbial contamination in the environment. This reinforces the concern that environmental reservoirs may serve as a role in of recurrent infections, antibiotic resistance, and the transfer of genetic material. There is a need for further studies on the regulatory mechanisms governing biofilm formation in E. coli, as well as effective methods for its prevention. The identification of biofilm-forming E. coli isolates in food and water samples in the present study underscores the potential risk these sources pose in the transmission of food- and waterborne illnesses. These findings highlight the critical importance of implementing stringent food safety protocols, regular monitoring, and maintaining proper hygiene practices to minimize E. coli contamination and reduce the risk of cross-contamination in both domestic and industrial settings.

5. Conclusion

The public health implications of these findings are significant. Environmental E. coli with biofilm-forming potential may act as persistent sources of infection, particularly for immunocompromised individuals. In addition, they present challenges for water sanitation, food safety, and infection prevention

strategies. Therefore, routine surveillance of environmental sources and assessment of biofilm-forming capacity in E. coli should be prioritized to prevent potential outbreaks and reduce the spread of resistant strains.

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