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Biochemical and Molecular Characterization of Biofilm Producing *Escherichia coli* Isolated from Clinical Specimens in Irrua Specialist Teaching Hospital, Irrua Edo State

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Abstract

Escherichia coli, like many bacteria with the ability to form biofilm, can withstand stress in its environment to evade the host defence mechanisms and even antimicrobial agents. The biochemical and molecular characterisation of biofilm-producing *Escherichia coli* isolated from clinical specimens within Ekpoma and its environs was analysed to isolate *E. coli*, detect their biofilm-forming capacity(s), and demonstrate their biofilm-forming and antibiotic resistance genes. Two methods were used to establish biofilm production, the Congo red agar (CRA) and microtitre plate methods. This gave a reproducibility of about 90%. Clinical isolates had stronger biofilm formers of 25.7% with a percentage of weak formers (17.1%). Antibiotic resistance was low before biofilm formation but increased by 61.5% after biofilm formation. Molecular characterisation tests revealed plasmid-encoded fimbriae H (fim h) and aggregate genes [(agg) 3IV] for biofilm formation for strong, moderate and weak formers only from both sources of isolates. The presence of plasmids for biofilm formation made multidrug resistance of commonly used antibiotics possible in their normal routine dosages. This was further established by the presence of antibiotic-resistance genes for quinolones and aminoglycosides. Biofilm-forming isolates were further tested with 75ul/g of acrydine orange (AO) dye for plasmid curing. Results showed a drop in biofilm formation from about 90% to 9.9%, and antibiotic resistance from 78.6% to 10.0%. Plasmid-mediated antibiotic resistance can be attributed to the widespread use of antimicrobial agents and self-medication which is of great concern for public health and a great threat to chemotherapy and medicine worldwide.

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1. Introduction

In microbiology, biofilm is defined as a community of microorganisms irreversibly attached to a surface and encased in an extracellular polymeric substance (EPS), with increased resistance to host cellular and chemical responses (Costerton *et al*., 1999). Biofilms are ubiquitous, and almost all, if not all, microorganisms have the ability(s) to form biofilms, but whether they would readily do so when provided with a non-conducive environment is an argument for another day (Costerton *et al*., 1999). Many bacteria form biofilms, including Gram-positive, e.g. Bacillus spp., Listeria monocytogenes, Staphylococcus spp., Lactic acid bacteria, e.g. Lactobacillus plantarum and Lactococcus lactis. Others are Gram-negative spp. like *E. coli* and Pseudomonas aeruginosa, (Singh *et al*., 2013). Biofilms constitute a protected mode of growth that allows them to survive in a hostile environment. The structures that form biofilms contain certain channels in which nutrients circulate (DeBeer *et al*., 1994), and cells in regions of a biofilm produce patterns of gene expressions (Davies & Geesey, 1995). Microorganisms undergo profound changes during their transmutation from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to environmental signals (O'Toole *et al*., 2000).

Escherichia coli a Gram-negative, aerobic, rod-shaped, coliform bacterium of the Genus Escherichia and the Family Enterobacteriaceae are enterics (Ekundayo *et al*., 2014). They are either non-motile or motile by peritrichous flagella, both fermentative and respiratory. Of the six Escherichia species, *E. coli* is the most common in human specimens and most commonly associated with human diseases (Isibor *et al*., 2013). *E. coli* with the K1 capsule are most frequently associated with neonatal sepsis and meningitis (Ekundayo *et al*., 2014). The organism *E. coli* is the type species, a laboratory specimen manipulated in all manner of microbiology and biotechnological research (Enabulele & Uriah, 2009). It has been extensively studied and has become a standard model system in Microbiology because it can be manipulated in all laboratory research (Reshes *et al*., 2008). It can live in environments with or without air, and it's found in the lower intestine of warm-blooded animals. The harmless strains are part of the microbiota of the gut of both humans and animals (commensal bacteria of humans and animals) they benefit their host by producing vitamin K2 which helps blood to clot and prevents colonization of the intestine with pathogenic bacteria, having a symbiotic relationship (Singh *et al*., 2013). *E. coli* has a strong biofilm-forming ability and is the most prevalent infecting organism in the family of Gram-negative bacteria (Costerton *et al*., 1999). *E. coli*, like most organisms, can attach themselves using their flagella and other appendages and subsequently attach themselves to a substrate in a microcolonial form known as biofilm. They produce a slimy substance known as extracellular polymeric substances or EPS. Biofilms are composed of one or more types of microorganisms attached to different surfaces

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Biofilms are composed of microcolonies of bacterial cells that are distributed in a matrix which consists of exopolysaccharides, proteins, salts, and cell materials 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 infections and inflammations (Frye & Jackson, 2013). Biofilms consist of multi-layered cell clusters which facilitate the adherence of these microorganisms to biomedical surfaces and protect them from the host immune system and antimicrobial therapy (Gara & Humphrey, 2001). In water, bacteria from different sources (human, animal and environment) can mix and resistance evolves resulting in the exchange and shuffling of genes, genetic platforms and vectors (Baquero *et al*., 2008). More than half of the infections caused by Pseudomonas aeruginosa, *Escherichia coli*, Vibrio cholerae and other bacteria involve biofilms (Protera, 1999; Iyevhobu *et al*., 2022).

Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents and the potential for these organisms to cause infections with indwelling medical devices (Dolan, 2002). The occurrence of biofilms on medical devices has come from studies in which the devices were examined upon removal from patients or were tested on animals or laboratory systems (Raad, 2010).

Biofilm formation occurs by at least three mechanisms: the redistribution of surface-attached, but motile cells, the multiplication of attached cells, and the recruitment of cells from the bulk fluid. These mechanisms depend on the organisms involved, the substratum and the environmental conditions (Costerton *et al*., 1999), the maturation of biofilm results in a complex architecture influenced by several biological factors and hydrodynamic features. The biological factors include cell-cell signalling between the biofilm bacteria, and possible competition or cooperation between the bacteria (Stoodley *et al*., 2002).

Biofilm production is a marker of clinically relevant infections (Svjetlana & Jasmine, 2007). Previous observations have confirmed that biofilms are not only resistant to antibiotics but a variety of disinfectants (Chen & Wen, 2011),

Many in vitro methods are used to detect and quantify biofilms isolated from specimens to provide the true picture of biofilm colonisation, and thus study their biochemical and molecular characteristics. Conventional biochemical tests using standard microbiological techniques will be used (Cheesbrough, 2008). The Microtitre plate method of O'Toole (2012), a quantitative method, and the Congo red agar (CRA) by Freeman *et al*. (1989), a qualitative method, will be carried out for the detection of slime production in *Escherichia coli*.

The scope of this study entails the isolation of *Escherichia coli* from clinical specimens. Pure isolates of *E. coli* were subjected to biofilm-forming assays, using qualitative and quantitative (Congo red agar and Microtitre methods, respectively). After which, biofilm-forming isolates in their various capabilities were subjected to antibiotic susceptibility testing for resistance and sensitivity patterns. Multiple drug resistance will be detected in biofilm-forming strains of

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isolates. This was followed by molecular characterisation of biofilm-forming *E. coli* and the detection of biofilm-forming and antibiotic resistance genes. This research aimed to isolate *E. coli* from clinical specimens, detect their biofilm-forming capacity(s), and demonstrate their biofilm-forming and antibiotic-resistance genes.

2. Method

2.1 Study Area

Clinical samples of urine, wound and stool were obtained from presumptive *Escherichia coli* isolates from a bank stock of isolates in Irrua Specialist Teaching Hospital (ISTH) Irrua, Edo State. An ethical approval for the clinical samples was obtained from the Ethics Committee of ISTH.

2.2 Sample size

The Sample Size Calculator.calculator.net. (2017) was used to determine sample size for this study.
The formular is; n=(Z^2 x p⊥^ (1-p⊥^))/ϵ^2
Where n=sample size from an infinite population size
Z=1.96, p⊥^=0.5, 3=0.05
Therefore, for this study, the sample size,
n=(1.96)^2×0.5(1-0.5)=3.8416×0.25=384.160.05
This was approximated to 400

2.3 Sterilisation of Media and Materials

All glassware was washed and rinsed with clean water, dried and then plugged with cotton wool and wrapped with foil paper before sterilisation in a hot air oven at 160°C for one hour. Wire loops were sterilised by dipping in ethanol, followed by flaming to red hot over a Bunsen flame (Cruickshank *et al*., 2000). All media were measured and sterilised using the autoclave according to manufacturers' instructions.

2.4 Procedures for Sample Collection:

Clinical Specimens: Isolates of *E. coli* from stool, urine and wound samples were randomly selected from the stock culture of isolates in ISTH. Isolates were processed by previously described methods (Cheesbrough, 2008) and were revived by inoculation onto Brain Heart Infusion (BHI) broth. The isolates were incubated at 37 °C for 24 hours and subcultured onto MacConkey and eosin methylene blue (EMB) agar. These were then incubated overnight at 37 °C. Morphological characteristics of isolates on these media were used to presumptively identify *E. coli* isolates before biochemical characterisation using the methods of Cheesbrough (2008).

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2.5 Microbiological Investigations

Isolation and identification of organism: Presumptive clinical isolates from urine, stool and wound swabs were retrieved from their storage slants by inoculating onto brain heart infusion broth, this is to ensure a rich harvest of viable cells. They were then incubated at 37 °C for 24 hours. They were then inoculated from the broth onto MacConkey agar and incubated at 37 °C for 24 hours (Iyevhobu *et al*., 2023)

2.5.1 Confirmation of E. coli isolates: presumptive *E. coli* colonies were further streak-inoculated onto EMB agar, which is selective for *E. coli* and incubated at 37°C for 24 hours. Metallic green sheen colonies were further subjected to biochemical tests for confirmation. Pure and confirmed *E. coli* isolates were inoculated into prepared slants and incubated at 37 °C for 24 hours. They were then preserved in the refrigerator for subsequent use (Isibor *et al*., 2013)

2.6 Biofilm Formation Assay

Two methods were used: the Congo red agar (CRA) plate method, a qualitative method described by Freeman *et al*., (1989) and the microtitre tube method (otherwise known as the 96 flat-bottom tube), a quantitative method as described by O'Toole (2012).

2.6.1 Congo red agar method (CRA): Materials used were brain-heart infusion broth (Oxoid, UK, 37g/L, sucrose 50g/L, Agar 1 (Oxoid, UK) 10g/L and Congo red indicator (dye) (Oxoid, UK) 8g/L. These were measured and prepared according to the manufacturer's instructions. Congo red was prepared separately from other medium constituents as a concentrated aqueous solution, autoclaved at 121 °C for 15 minutes. This was added to autoclaved brain heart infusion agar with sucrose at 55 °C. They were allowed to cool and poured into sterile Petri dishes and allowed to solidify. Test organisms from samples were then inoculated onto CRA plates, labelled and incubated overnight at 37 °C. After 24 hours, growths were observed and recorded. This experiment was performed in triplicate as described in the protocol. Grey to black colonies with dry crystalline consistency were indicative of biofilm formation, a pink to a darkening and red colony with the absence of a dry crystalline colonial morphology was indicative of intermediate and non-biofilm forming results, respectively. (Naveen *et al*., 2014)

2.6.2 Microtitre plate method: This quantitative method was described as the gold standard for biofilm detection by Christensen *et al*., (1998), a more recent and modified method by O'Toole (2012) for biofilm detection in Pseudomonas aeruginosa (but not limited to it).

2.6.3 Materials: 96-well flat-bottom tubes, 0.1% crystal violet solution, 30% acetic acid and paper towels.

(1) Growth of a biofilm: A culture of *E. coli* isolates from clinical sources was grown in a low bile medium (sterile water plus 5% tryptophan broth) and incubated at 37 °C for 24 hours. This is to allow for a robust harvest of test organisms. Overnight growth culture was then

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diluted 1:100 into the fresh medium of low bile in test tubes for biofilm assay. From these test tubes, 100 µL of the diluent was introduced into the 96-well dish. This was done in four replicate wells for each isolate taken randomly from clinical sources. Petri dishes were then incubated at 37 °C for 24 hours. (Naveen *et al*., 2014)

(2) Staining of the biofilm: After incubation, plates were turned over and shaken to remove the liquid. Each plate was then submerged in a tub of distilled water. This was repeated twice to remove unattached cells and media components that could be stained together with attached cells, to give erroneous results. 125µl of 0.1% crystal violet (CV) solution was then added to each well of the triplicate wells. This step was done with all necessary precautions since CV is hydroscopic. Plates were then incubated by covering with sterile filter paper at room temperature for 10- 15 minutes. Plates were then rinsed 3-4 times by submerging in a tub of sterile water. They were shaken and blotted vigorously on sterile paper towels to remove excess cells and dye. Microtiter plates were then turned upside down to dry for 12 hours and were photographed for qualitative assay. (Mathur *et al*., 2006)

(3) Quantifying the biofilm: 125ul of 30% acetic acid solution was added to each tube to solubilise the CV. It was then incubated at room temperature for 10- 15 minutes. Absorbance quantity (optical density of cells) in the plates was done using estimated values from charts prepared by Hassan *et al*., (2019). Sterile water in the plates was used as a negative control, while a strain of ATCC 25922 was used as a positive control. The optical density of cells was calculated thus: (ODc = Optical density cut-off value).

(4) It is the average OD of the negative control +3 x standard deviation (SD) of the negative control, where the OD cut-off value is 580µm to 620µm. The calculation was done with the estimated Eliza card reader plate to get results for 'strong,' 'moderate,' 'weak' and 'non-biofilm formers' at 580µm wavelength.

2.7 Curing of biofilm-producing E. coli isolates using acridine dye

was carried out to determine if the genes responsible for biofilm formation in clinical and environmental isolates were plasmid or chromosomal-mediated.

Procedure: 75µl/g of acrydine dye was used in this protocol because it is safer on bacterial cells in such concentrations than ethidium bromide (Inyang *et al*., 2015). The acrydine dye was measured and sterilised according to the manufacturer's instructions, and mixed with already sterilised nutrient agar prepared according to the manufacturer's instructions. The mixture was then poured onto plates and allowed to solidify. Colonies of isolates that formed biofilms strongly, moderately and weakly on CRA from both clinical and environmental sources were sub-cultured onto freshly prepared nutrient agar plates incubated at 37°C for 24 hours, for purification. Isolates were then subjected to biofilm assay using the microtitre tube method.

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2.8 Molecular identification of biofilm-producing E. coli isolates

Clinical isolates that formed biofilm strongly, moderately and weakly were randomly harvested from their respective bijou bottles to establish the presence of two sets of genes.

1) Genes for biofilm formation, which are [fimbriae genes (fim H) responsible for adhesion and aggregate genes (agg (IV)3)] responsible for colony formation.
2) Genes responsible for antibiotic resistance, which are quinolones and aminoglycosides.

The Zymo Research Quick DNA fungal/ bacterial miniprep kit purchased from South Africa through registered and accredited agents was used, and experiments were carried out in the Microbiology laboratory of ISTH, Irrua, using the manufacturer's protocol.

For the targeting of fim H genes, the primer sequence (5' to 3') used was TGC AGA ACG GAT AAG CCG TGG/GCA GTC ACC TGC CCT CCG GTA (Johnson and Stell, 2000).
For the targeting of antibiotic resistance gene (quinolones, qnrB), the primer sequence (5'to 3') used was ATG AGC GAC CTT GCG AGA G/TGG TTG CCA TAC CTA CGG (Cavaro *et al*., 2011).

For the targeting of antibiotic resistance genes (aminoglycosides, aac(3)IVa), the primer sequence (5' to 3') used was GTG TGC TGC TGG TCC ACA GC/AGT TGA CCC AGG GCT GTC GC (Otto, 2013).

2.9 Statistical Analysis

The statistical analysis was done using Analysis of Variance (ANOVA) (Soper, 2019) and Data summary and graphical illustration (Statistical Package for Social Science, 2017).

3. Results

Table 1: Prevalence of *E. coli* Isolates from Clinical Isolates in the study area.

Clinical Isolates	ISTH Irrua	Total	Percentage
Urine	29	29	41%
Wound	21	21	30%
Stool	20	20	29%
Total (%)	70	70	100%

The number of *Escherichia coli* isolates from the specimens was seventy as represented in Table 1. Urine specimens had the highest prevalence rate of *E. coli* isolates 29(41.3%), followed by wound 21(30.0%) and the least was from stool 20 (28.6%) among the clinical specimens.

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Table 2: Biochemical Identification of *Escherichia coli*

Tests	Clinical Samples
Motility	Positive
Gram Stain	Negative Bacilli
Catalase	Positive
Citrate	Negative
Indole	Positive
Oxidase	Negative
Urease	Negative
KIA	Yellow/Yellow/Gas

Biochemical identification of *E. coli* isolates was confirmed using standardised confirmatory tests as laid down by the NCCLS guidelines. The Gram stains for all isolates revealed Gram-negative bacilli. Results for motility, catalase and indole were positive, while nitrate, oxidase, and urease tests were negative. The result for the KIA test came out positive (yellow/yellow/gas production) to confirm the organism as *E. coli* (Table 2).

Table 3: Rates of biofilm formation by *E. coli* from clinical samples

Clinical isolates	Strong formers (%)	Moderate formers (%)	Weak formers (%)	Non-formers (%)	Total isolates (%)
Microtitre Plate	22(31.4)	21(30.0)	14(20.0)	13(18.6)	70(100.0)
CRA	18(25.7)	23(32.9)	12(17.1)	17(24.3)	70(38.5)

Note: For clinical specimens, a total of 57(81.4%) isolates formed biofilm while 13(18.6%) did not;

Biofilm assay was done on all isolates using the CRA and microtitre plate (96 flat-bottom tube) methods. All isolates responded in varying capacities as strong, moderate, weak and non-biofilm formers. On CRA plates, 53(75.7%) formed biofilm out of the 70 clinical isolates, while 17 (24.3%) did not (Table 3). On the other hand, out of the 70 clinical isolates, 57 (81.4%) formed biofilm, while 13 (18.6%) did not.

Table 4: Biofilm formation and Optical Density (OD) range on microtitre tubes

Average OD Range (µm)	Biofilm capacity	Number of clinical isolates
2,320	Strong Formers	22(31.4%)
1,160	Moderate Formers	21(30.0%)
>=0,580	Weak Formers	14(20.0%)
<=0,580	Non-Formers	13(18.6%)

Note: Total of 57(81.4%) isolates formed biofilm while 13(18.6%) did not

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Using the microtitre tube method, 154 (84.6%) isolates formed biofilm, with an optical density (OD) wavelength range of between ≤0,580µm to 2,320µm, ranging from weak to strong formers, while 28 (15.45) recorded OD wavelength range of≥0,580µm, because they could not form biofilm, thus could not be picked up by the light (Table 4).

4. Discussion

This study was designed to isolate and identify *E. coli* isolates from clinical samples and detect their biofilm-forming capabilities. Seventy (70) clinical isolates were identified in the present study. The analysis and purification of the 70 clinical isolates collected from the Microbiology Department of ISTH, n=70, with stool recording 20 (29.0%), urine 29 (41.0%), and wound 21 (30.0%).

Earlier reports have shown that *E. coli* can be isolated from various clinical sources, including wounds, stool (Isibor *et al*., 2013), and urine (Oriomah & Akpe, 2019). The prevalence of *E. coli* in various specimens varies significantly across different parts of the globe, ranging from 12.8% in Canada (Zhanel *et al*., 2008) to as high as 70.6% in Iran (Virginio & Soto, 2020). Van Ellias *et al*. (2011) reported a high incidence rate of *E. coli* in India, while in another study from Kaduna, Nyandjou *et al*. (2018) recorded a low incidence rate of *E. coli*.

A biofilm is a community of microorganisms and extracellular polymers attached to a surface (Pfaller, 1996). The ability to form biofilms is associated with pathogenicity and should be considered an important determinant of virulence during infections (Naveen *et al*., 2014).

This study isolated *E. coli* from three clinical sites, and to establish a baseline for comparison, a biofilm assay was carried out on all isolates using two methods for biofilm detection: the CRA and the microtitre tube methods. The results showed that 18 (25.7%) isolates were strong biofilm formers, 23(32%) moderate formers, 12(17.1%) weak biofilm formers 17(24.3%) non-biofilm formers on CRA plates (Table 3). This agrees with other reports by Zhanel *et al*. (2008), who reported that clinical samples formed a larger percentage of strong biofilm-formers. The rate of biofilm formation can be said to be high in clinical isolates.

Other studies have shown that the CRA method can determine biofilm formation. Naveen *et al*. (2014) reported that out of 120 Candida spp. evaluated, 46 (38.3%) produced biofilms, with 10 (21.7%) being strong and 36(78.3%) being weak, while 74 (61.7%) did not form biofilms. In addition, Vinitha and Ballal (2011), in a similar study, reported that 81 (73%) out of 111 Candida spp. produced biofilms using the CRA method. These reports concluded that the CRA method is fast, practical, and reliable for biofilm detection in microorganisms. In contrast, the results recorded for the strong formers using the microtitre tube method were 22 (31.4%), 21 (30.0%), and 14 (20.0%), respectively. It was 13 (18.6%) for non-formers.

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In a study conducted by Netsanet *et al*. (2017) using the microtitre tube method, P. aeruginosa and Proteus spp. were 100% biofilm formers, followed by *E. coli* (77.8%), and S. aureus (80%). Similar patterns of biofilm formation have been reported in studies conducted in India (Ponnuasamy *et al*., 2012). In contrast, a work carried out in Egypt reported that Klebsiella spp. produced higher levels of biofilms. (44.4%), and S. aureus (42.9%) than in *E. coli* (31.6%) (Iyevhobu *et al*., 2023).

Furthermore, the calculation of the optical density (OD) value of cells shows that the greater the adherence of cells, the higher the OD value. Although the recommended OD value range is between 540µm and 620µm, in this study, an estimated OD wavelength range of 580µm was used according to the calculations of Hassan *et al*. (2019). The results of this study showed that 28 (15.4%) non-formers and 52 (28.6%) weak formers had an OD value range of≥0,580µm, 55 (30.2 %) moderate formers had an OD value of 1,160µm, and 47 (25.8 %) strong formers had an OD value of 2,320µm (Table 4). It is pertinent to note that with an increase in the capability of biofilm formation, there is a corresponding increase in the OD value. These results are consistent with those of Mathur *et al*. (2006), who reported an increase in OD values in their study.

In a comparison of the sensitivity of these two methods, following the demonstration of biofilm formation among isolates, it was observed that the reproducibility of both methods was above 80%. Although there was no standardised control for the CRA method, the sensitivity of biofilm formation was observed to be the same as that indicated in the data recorded when compared to that of the microtitre tube method, which had a standardised control. The sensitivity may be the same because of the problem of different individual interpretations of the indicated colours. The standard used in this study was an in-house standard from ISTH Irrua.

Other reports have documented in their study, using various biofilm assay methods that the 'gold standard' method for biofilm formation in bacterial cells is the microtitre tube method, since it measures the effects of agents against biofilm formation produced on walls of wells, as against that of CRA that just takes up the colour of the dye (Mathur *et al*., 2006).

Other researchers recommended the microtitre tube method to be superior and more accurate than the CRA method based on parameters such as sensitivity, specificity, negative predictive value, positive predictive value, and accuracy, which were not considered in this study. True positives were biofilm formers by the microtitre and CRA methods, while false positives were biofilm formers by the CRA method only. False negatives were isolates which were non-biofilm producers by CRA but were producing biofilms by the microtitre method. For them, true negatives were those which were non-biofilm formers by all methods, but biofilm formers by the same methods (Afreenish *et al*., 2011). Some authors concluded that the microtitre tube method and the tube method correlate well for strong biofilm formers, but the same cannot be said for the CRA method because their observations were in disagreement with those of Freeman *et al*. (1989). As such, they recommended that the microtitre method is more accurate and reproducible, as indicated by their data, which is in line with the results of this study.

E. coli-like bacteria have the ability(s) to form biofilms, withstand stress in their environments, and evade host defence mechanisms and even antimicrobial agents (Wood *et al*., 2008).

This study has shown that *Escherichia coli* can be isolated from almost anywhere that microorganisms can grow, as long as the conditions for growth and survival are favourable for it. This is supported by the fact that *E. coli* was isolated from all clinical specimens used in this study, and the isolates were also able to form biofilms at different capacities using both colourimetric and cell adherence (microtitre/optical density measurement) methods.

5. Conclusion

Ideally, the physiological and genetic engineering programming of bacteria expresses the desired phenotype (the observable physical character–genetically and environmentally determined qualities of an organism) under physiochemical circumstances in which there is little or no control. Based on these results, further studies should be conducted on biofilm-forming for better understanding.

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Availability of Data and Materials

The authors declare that they have no conflicts of interest.

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Competing Interests

The authors declare no conflicts of interest

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