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Biochemical and Molecular Characterization of Biofilm Producing Escherichia Coli Isolated from Environmental Specimens within Ekpoma and its Environs

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ABSTRACT

A community of bacteria that are permanently adhered to a surface and covered with extracellular polymeric substances (EPS) and have heightened resistance to host cellular and chemical reactions is referred to as a biofilm in microbiology. One or consortium of microorganisms affixed to various surfaces make up biofilms. This study analyzed the biochemical and molecular characterization of biofilm producing Escherichia coli isolated from environmental specimens within Ekpoma and its environs to separate the ambient E. coli specimens, identify the biofilm-forming capacity or capacities, and show the antibiotic resistance and biofilm-forming genes. In ambient samples, Escherichia coli was recovered with a prevalence of 112. Biofilm generation was demonstrated using two techniques: the microtitre plate method and the Congo red agar (CRA) method. This resulted in a 90% re-producibility. There were a lot of weak formers (41.1%) among the environmental isolates (20.5%). The results of the statistical analysis indicated that there was no significant difference between the two approaches, with the p-value being 0.167369 and $p \geq 0.05$. Prior to biofilm formation, antibiotic resistance was modest (65.2%), but it increased after biofilm formation. Plasmid-encoded fimbriae H (fim h) and aggregation genes [(agg)3IV] for biofilm formation for strong, moderate, and weak formers alone from both isolate sources were constituted by molecular characterization test. Multidrug resistance of routinely used antibiotics in their typical routine dosages was made possible by the inclusion of plasmids for biofilm development. The existence of antibiotic resistance genes for aminoglycosides and quinolones served as additional evidence of this. These can be investigated further with the goal of preventing the formation of biofilm by infection-associated cells. Acrydine orange (AO) dye at a concentration of 75 ul/g was used to evaluate biofilm-forming isolates for plasmid curing. The findings revealed a decrease in antibiotic resistance from 87.5% to 6.3% and biofilm development from roughly 90% to 9.9%. Plasmid-mediated antibiotic resistance is a major public health concern and a serious threat to chemotherapy and medicine worldwide.

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Introduction

Although practically all bacteria, if not all of them, have the capacity to create biofilms, it is debatable whether they would do so readily in an unfavourable environment. Biofilms are common in nature [1]. Gram-positive bacteria like Bacillus species, Listeria monocytogenes, and Staphylococcus species, as well as lactic acid bacteria like Lactobacillus plantarum and Lactococcus lactis, are among the many types of bacteria that can produce biofilms. Some are Gram-negative bacteria, such as Pseudomonas aeruginosa and E. coli [2]. At least three processes contribute to the creation of biofilms: recruiting cells from the bulk fluid, multiplying attached cells, and redistributing surface-attached yet motile cells. The species involved, the substrate, and the surrounding circumstances all influence these processes [1]. Numerous biological elements and hydrodynamic characteristics affect how a biofilm matures and develops a complex architecture. Cell-cell communication among the biofilm bacteria and potential bacterial cooperation or competition are examples of biological variables [3]. In order to thrive in a harsh environment, biofilms provide a protected development mode. Cells in various areas of a biofilm display distinct patterns of gene expression, and the structures that make up biofilms have specific channels that allow nutrients to flow. It is evident that when microorganisms transition from planktonic (free-swimming) creatures to cells that belong to a

complex, surface-attached community, they go through significant modifications. These modifications take place in response to various environmental cues and are manifested in the novel phenotypic traits that biofilm bacteria have developed.

E. coli is the most common infecting organism in the Gram-negative bacterial family and possesses a great capacity to build biofilms. Like the majority of organisms, E. coli can adhere to one another via its flagella and other appendages. It can then adhere to a substrate in a form known as biofilm, which is a microcolony. They accomplish this by releasing extracellular polymeric substances, or EPS, a slimy material.

The microcolonies of bacterial cells that make up biofilms are dispersed over an aqueous matrix made up of proteins, salts, exo-polysaccharides, and cell components. About 85% of a biofilm's volume is made up of the matrix. According to reports, the most frequent cause of chronic infections and inflammations is bacterial biofilms. The multi-layered cell clusters that make up biofilms let these microorganisms attach to biomedical surfaces while shielding them from the human immune system and antimicrobial treatment. The emergence and spread of bacterial resistance to widely used antibiotics are causes for concern on a global scale. Bacteria from human, animal, and environmental sources can mingle in water, and resistance develops as a result of the promiscuous exchange and rearranging of genes, genetic platforms, and genetic vectors. According to, biofilms are involved in over 50% of the infections brought on by Pseudomonas aeruginosa, Escherichia coli, Vibrio cholerae, and other bacteria [4].

Microbial adhesion and biofilms are significant from an industrial perspective in addition to a medical one, particularly in the food sector where they are found on a wide range of surfaces that come into contact with food. Bacteria can sense surfaces, and when they come into touch with them, a sophisticated differentiation mechanism that produces alginate is triggered. About 15 minutes after attachment, it was demonstrated that Pseudomonas aeruginosa had up-regulated genes required for alginate synthesis. The biofilm cells differ from their planktonic counterparts in terms of phenotype and metabolism due to a variety of modifications in gene regulation. Bacillus cereus, Pseudomonas putidia, and Escherichia coli have all convincingly demonstrated this differentiation. Like many other bacteria that may form biofilms, Escherichia coli can tolerate environmental stressors to avoid host defense mechanisms and even antimicrobial drugs [5]. It is important to realize that bacteria that can form biofilms can either be harmful or non-harmful, as is the case with E. coli.

Genes and regulatory circuits crucial for early cell-surface contacts, biofilm maturation, and the reversion of biofilm microorganisms to a planktonic mode of growth have been discovered by recent genetic and molecular methods used to research bacterial and fungal biofilms. Thus far, research indicates that the process of planktonic-biofilm transmutation is intricate and tightly controlled. Numerous severe illnesses linked to biofilms have been studied. Some may be caused by a combination of biofilm microorganisms, while others are solely caused by bacterial or fungal biofilms. The following are examples of biofilm-associated microorganisms that are frequently isolated from indwelling medical devices and the organisms that cause them:

The formation of biofilms is thought to be an indicator of infections that are clinically significant. Prior findings have demonstrated that biofilms are resistant to a range of disinfectants in addition to antibiotics, underscoring the significance of characterizing them in infection prevention. The age of the biofilm may affect susceptibility. The issue will unavoidably worsen and the aged biofilms will become more challenging to eradicate if an indwelling medical device becomes colonized by a biofilm. Although there are numerous methods for studying biofilms, it is essential to create standardized methods. Numerous laboratories have standardized a range of techniques, each with unique advantages and disadvantages. It has been demonstrated that older biofilms are even less vulnerable to antimicrobial treatments than younger ones. Furthermore, the likelihood of resistance-plasmid transmission may rise with time if the biofilm contains organisms with acquired resistance.

To get a realistic picture of biofilm colonization and investigate its biochemical and molecular properties, a variety of in-vitro techniques are employed to identify and measure biofilms that have been separated from specimens. The normal microbiological methods and routine biochemical testing will be employed. The Congo red agar (CRA), developed by, and the microtitre plate method of, which is a quantitative method, will be used to identify slime generation in Escherichia coli [6].

Infections linked to biofilm-forming bacteria and bacteria in biofilms have become a major global health and medical concern. Concerns and issues about rising antibiotic resistance have been noted and documented over the years. The rise of drug-resistant microorganisms in human medicine, the careless use of antibiotics, and complicated socioeconomic behavioural antecedents are the causes of this growth. Antibiotics used to treat bacterial infections at standard concentrations are no longer producing the desired results. It seems as though treatment is going more slowly than anticipated and, in certain situations, not at all, even when dosages are raised and antibiotics are taken together. Knowing the diagnosis of an infection helps the patient feel less stressed and spends less money, while also making treatment and recovery easier for the doctor. By acting as a selective pressure, antimicrobials themselves enable the development of resistant bacteria inside of sensitive counterparts that may exhibit the same drug resistance in donor strains. Antibiotic-resistant genes on bacterial plasmids have further aided in the spread of multidrug-resistant bacteria, which is concerning given the rise in their development in recent years. As a result, first-line antimicrobials are gradually disappearing, and many antimicrobial agent classes have decreased in effectiveness. In managing infectious infections, this has posed a significant clinical challenge. The degree to which an organism can fight itself against an antimicrobial agent when in a biofilm phase will be a valuable piece of information to aid a patient and a doctor in a long-term healing process. Eliminating the plasmids that cause antibiotic resistance and biofilm development is crucial for microbial genetics and chemotherapy. Thus, the purpose of this work is to examine the production of biofilms in E. coli from environmental specimens, identify the genes responsible for antibiotic resistance, and comprehend the interrelationships between these genes.

Numerous bacteria, including E. coli, have been subjected to the microtitre plate assay for biofilm development. According to the microtitre plate method is a straightforward technique that may be readily adapted to assess several strains or growing conditions in a single experiment. Although they called it the slime generation assay, also used the Congo red method (CRA) to perform biofilm development in their findings. They found differences in the ability of E. coli isolates to produce biofilm using the CRA and microtitre plate methodologies. Their investigation was conducted to evaluate in-vitro biofilm development. Based on their research, 12 (85.6%) of the 14 isolates examined developed biofilm on CRA, and 2 (14.4%) were negative; 10 (71.4%) developed biofilm on microtitre plates,

and 4 (28.0%) were negative. According to, out of 110 isolates examined for biofilm formation using the microtitre plate assay, 25 were characterized as strong formers, 45 as moderate formers, and 40 as non-formers [7]. S. aureus (11.4%), E. faecalis (4.2%), P. aeruginosa (4.2%), K. pneumoniae (15.7%), E. coli (27.1%), and S. epidermidis (37.1%) accounted for the majority of the organisms linked to biofilm formation. The microtitre plate assay revealed that 40 (36.3%) were non-formers and 70 (64.7%) were biofilm formers. They came to the conclusion that the microtitre techniques were a more accurate and quantitative way to find out when bacterial cells were forming biofilms. Additionally, regional data from India revealed that, of the 152 isolates examined, 53.9% produced biofilms using the microtitre method, while 46% did not [8]. reported in another investigation that of 147 isolates of S. epidermidis, 79 (53.7%) had biofilm development detected by tube method (TM), and 64 (43.5%) had CRA. They demonstrated that TM outperforms CRA in the identification of biofilm. In their investigation, did not suggest the CRA approach for biofilm identification. In contrast to the microtitre approach, which identified 57.1% of the 128 S. aureus isolates as biofilm bacteria, CRA only identified 3.8% of them as biofilm formers. The tube method was employed by to identify the production of biofilms among uropathogens. Their findings showed that biofilm development was present in 75% of the isolates. 99 were determined to be non-formers and 1 to be biofilm formers using the CRA approach. The CRA method's sensitivity (11%), specificity (92%), and accuracy (41%), as well as its correlation with other methods, were all quite poor. Three isolates were determined to be false positives and sixty-two to be false negatives using this method. According to, the microtitre approach can be suggested as a universal technique for detecting bacteria that produce biofilms in lab settings when contrasted with TM and CRA methods [6].

This study's scope includes isolating Escherichia coli from environmental specimens in the communities of Ekpoma, Iruekpen, Opoji, and Irrua, all of which are located in the Esan West Local Government Area of Edo State. Following that, pure isolates of E. coli will undergo biofilm-forming experiments utilizing both qualitative and quantitative techniques (Microtitre and Congo red agar, respectively). The isolates that form biofilms in their different capacities will then be tested for antibiotic resistance and sensitivity patterns. Biofilm-forming isolate strains also show multiple medication resistance. The molecular characterisation of E. coli that forms biofilms and the identification of genes linked to antibiotic resistance and biofilm formation was determined the overall goal of this study is to separate the ambient E. coli specimens, identify the biofilm-forming capacity or capacities, and show the antibiotic resistance and biofilm-forming genes.

Materials and Methods

Study Area

In Ekpoma, Iruekpen, Opoji, and Irrua, environmental samples were collected from abattoirs, cattle dung, chicken droppings, toilet seats, door handles, run-off waters, and dumpsites. The ISTH Ethics Committee provided ethical approval.

Sample Size

The Sample Size Calculator.calculator.net. (2017) was used to determine sample size for this study.

$$\text{The formular is; } n = \frac{Z^2 \times \hat{p}(1-\hat{p})}{\epsilon^2}$$

Where n=sample size from an infinite population size

$$Z=1.96,\ \hat{p} = 0.1,\ \epsilon=0.05$$

Therefore, for this study, the sample size,

$$n=(1.96)^2\times0.1(1-0.1)=3.8416\times0.09=138.3$$

This was approximated to 138 ±20

Sterilization of Media and Materials

All glassware were first cleaned, rinsed with fresh water and dried. They were then plugged with cotton wool and wrapped in foil paper before sterilizing for an hour at 160°C in a hot air oven. Wire-loops were disinfected by soaking in ethanol and then burning them to red heat over a Bunsen flame. Media used were prepared following manufacturers' instructions.

Procedures for Sample Collection

Sterile swab sticks were used to collect samples from garbage sites, toilet seats, and door handles. Following a rainfall, capped sterile universal bottles were used to collect run-off water samples, primarily from the waters surrounding dumpsites. Abattoir specimens were collected 12 hours after cattle were killed and disposed of, right before the facilities were cleaned and sanitized for the day. Poultry droppings were collected from the birds halfway before they fell to the ground using swab sticks. Cattle excrement was collected from various cows at various sampling locations using the same process. In order to prevent contamination from the ground in the event that dung or droppings reached the ground prior to collection, the specimen was collected halfway through. All specimens were immediately taken to the Microbiology laboratory of Ambrose Alli University, Ekpoma for analysis.

Microbiological Investigations

Isolation of Organism

The organisms were released by dipping swab sticks of each of the environmental specimens from abattoirs, toilet seats, dumpsites, door handles, chicken droppings, and cattle dung in 5ml of distilled water and vortex for 1min. The specimen was then streaked on previously prepared media plates of MacConkey agar using a sterile wire loop after the swabs had been used to create an inoculum well. The plates were then incubated for 24 hours at 37 °C. Universal bottles were used to inoculate MacConkey plates with run-off water specimens, which were then incubated for 24 hours at 37 °C. The plates were checked for the distinctive halo pink colonies that are characteristic of lactose-fermenting Escherichia coli.

Confirmation of E. coli isolates: The halo pink colonies were further streak-inoculated onto EMB agar and incubated for 24 hours at 37 °C. Colonies with a metallic green sheen underwent further biochemical testing to confirm the isolates. **Pure colonies of Esch coli were then inoculated onto already prepared Nutrient agar slants, incubated for 24 hours at 37 °C and stored in the refrigerator until required for use**. E. coli isolates were biochemically identified using the techniques outlined by.

Biofilm Formation Assay

Two techniques were employed: the Microtitre tube method (sometimes referred to as the 96 flat bottom tubes), a quantitative technique as explained by, and the Congo red agar (CRA) plate method, a qualitative technique as described by.

Congo Red Agar Method (CRA)

Oxoid, UK's brain-heart infusion broth (37g/L), sucrose (50g/L), Agar 1 (10g/L), and Congo red indicator (dye) (8g/L) were the materials utilized. All of these were prepared and measured in accordance with the guidelines provided by the manufacturer. Congo red was made as a concentrated aqueous solution and autoclaved for 15 minutes at 121 °C, apart from the other medium ingredients. This was then added to sucrose-containing autoclaved brain-heart infusion agar at 55 °C. After cooling, they were transferred into sterile Petri dishes and left to set. Samples' test organisms were subsequently inoculated onto CRA plates, labeled, and incubated at 37 °C for the entire night. Growths were noted and examined after a 24-hour period. According to the protocol, this experiment was carried out in triplicate. Biofilm formation was indicated by grey to black colonies with dry crystalline consistency, whereas intermediate and non-biofilm forming findings were shown by pink to darkening and red colonies without a dry crystalline colonial morphology.

Microtitre Plate Method

A more current and improved method by for biofilm identification in Pseudomonas aeruginosa (but not exclusively) was employed for this work. described this quantitative method as the gold standard method for biofilm detection.

Materials needed were: Paper towels, 0.1% crystal violet solution, 30% acetic acid, and 96 well flat-bottom tubes were required.

Growing of a Biofilm

In a low bile medium (sterile water plus 5% tryptophan broth), a culture of E. coli isolates from environmental sources was cultivated and cultured for 24 hours at 37 °C. This will enable a healthy harvest of test organisms. For the biofilm assay, the overnight-grown culture was subsequently diluted 1:100 into new low-bile media in test tubes. 100ul of the diluent was added to the 96-well plate from these test tubes. For every isolate selected at random from environmental sources, this was carried out in four replicate wells. After that, the dishes were incubated for 24 hours at 37 °C.

Staining of the Biofilm

To get rid of the liquid, plates were incubated, then flipped over and shaken. After that, a tub of distilled water was used to immerse each dish. This procedure was carried out twice in order to eliminate detached cells and medium elements that might stain with connected cells and produce inaccurate findings. Following that, 125 µl of a 0.1% crystal violet (CV) solution was added to each triplicate well. Given that CV is hydroscopic, every precaution was used throughout this process. After that, plates were left at room temperature for ten to fifteen minutes while covered with sterile filter paper. The plates were then immersed in a tub of sterile water and washed three or four times. To remove extra cells and dye, they were shaken and wiped vigorously on a stack of sterile paper towels. Following a 12-hour drying period, microtiter plates were inverted and photographed for the qualitative test.

Quantifying of the Biofilm

To dissolve the CV, 125ul of a 30% acetic acid solution was added to each microtitre tube. After that, it was incubated for ten to fifteen minutes at room temperature. Charts created by were used to quantify the absorbance quantity (optical density of cells) in the plates [9]. The ATCC 25922 strain served as the positive control, and sterile water in the plates served as the negative control. Cells' optical density was determined using the formula (ODc=Optical density cut off value).

(4) It is the average OD of the negative control plus three times the negative control's standard deviation (SD), with an OD cut-off value between 580 and 620 µm. Results for "strong," "moderate," "weak," and "non-biofilm formers" at 580µm wave length were calculated using an estimated Eliza card reader plate.

Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion method using Mueller-Hinton agar (Hi Media Laboratories, Mumbai, India) was employed in this work in accordance with the guidelines provided by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2006). The antimicrobial drugs that were evaluated included ceftazidine (10 ug), ciprofloxacin (5 ug), cefuroxidime (30 ug), cefixime (5 ug), gentamycin (10 ug), ofloxacin (5 ug), and augmentin (30 ug) in the appropriate quantities.

Procedure

In bijou bottles, test organisms were individually inoculated into 3 milliliters of sterile peptone water and then incubated for the entire night at 37 °C. Next, 0.5% MacFarland turbidity was used to standardize the turbidity of the overnight broth culture. After preparing nutrient agar plates, the test organism was inoculated onto bijou bottles filled with saline water. It was then let to stand for three to five minutes, and then gently rocked to guarantee that the test organism was distributed evenly throughout the saline water. The extra broth was then transferred to a jar for disinfection, and this was put onto the nutritional agar plates. A Whatsman filter paper impregnated with several antibiotics at varying concentrations was placed onto the agar plate's surface using sterile forceps. To make sure the filter disc sits properly and doesn't slip or fall off, it was gently pressed onto the agar surface using forceps. The plate cover was put back on and properly labeled. After that, they were incubated for 24 to 36 hours at 37°C. By measuring the zones of inhibition with a graduated ruler at the conclusion of the incubation period, the isolates' susceptibility to the antibiotics was ascertained. According to Cruickshank et al. (2000), zones with a diameter of 13 mm or more were considered sensitive, whereas those with a diameter of less than 13 mm were considered resistant to the antibiotics tested. As a positive control, ATCC 25922 strain was employed. Isolates were tested for antibiotic susceptibility prior to, during, and following the formation of biofilms as well as after being cured with acrydine dye.

Curing of Biofilm Producing E. Coli Isolates Using Acrydine Dye
This was done to find out if the genes that caused biofilm development in environmental isolates were chromosomal or plasmid mediated.

Procedure

Since acrydine dye is less harmful to bacterial cells at such concentrations than ethidium bromide, 75µl/g of it was utilized in this procedure. Following the manufacturer's directions, the acrydine dye was measured, sterilized, and combined with nutrient agar that had already been sterilized. After that, the mixture was put onto plates and left to set. For purification, colonies of isolates that formed biofilms on CRA strongly, moderately, and weakly were subculture onto newly made nutrient agar plates and incubated for 24 hours at 37°C. After that, isolates were put via the microtitre tube method of biofilm test.

Molecular Identification of Biofilm Producing E. Coli Isolates

In order to determine two groups of genes, the environmental isolates that formed biofilm vigorously, moderately, and weakly were randomly taken out of their respective bijou bottles.

- The adhesion-related [fimbriae genes (fimH) and the colony-forming [aggregate genes (agg (IV)3)] are the genes involved in biofilm development.
- Aminoglycosides and quinolones are the genes that cause antibiotic resistance.

In accordance with the manufacturer's protocol, studies were conducted in the ISTH, Irrua Microbiology Laboratory using the Zymo Research Quick DNA fungal/bacterial miniprep kit, which was acquired from South Africa through registered and accredited agents.
For the targeting of fim H genes, primer sequence (5' to 3') used was TGC AGA ACG GAT AAG CCG TGG/GCA GTC ACC TGC CCT CCG GTA.

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

Statistical Analysis
Analysis of Variance (ANOVA) (Soper, 2019), data summary, and graphical illustration (Statistical package for Social science, 2017) were used in the statistical analysis.

Results
The Ekpoma, Opoji, and Irrua populations provided 280 environmental samples, which included samples from the abattoir, cow dung, poultry droppings, dumpsites, rainwater, toilet seats, and door handles. One hundred and twelve (112) Escherichia coli isolates were found in the ambient sample (Table 1).

Table 1: Prevalence of E. coli Isolates from Sampling locations

Environmental Isolates	Ekpoma	Iruekpen	Opoji	Irrua Area	Total	Percentage
Abbatoir	3	2	6	5	16	14%
Cattle Dung	5	3	4	3	15	13%
Poultry droppings	12	8	7	5	32	29%
Toilet Seats	7	2	2	3	14	13%
Door Handle	3	2	2	1	8	7%
Dumpsite	6	2	2	1	11	10%
Run-Off Water	8	3	2	3	16	14%
Total (%)	44(39.3%)	22(19.6%)	25(22.3%)	21(18.8%)	112	100%

The prevalence rates of E. coli isolates from the environmental specimens and their locations were, 44 (39.3%) in Ekpoma, 22 (19.6%) in Iruekpen, 25 (22.3%) in Opoji and Irrua had 21 (18.8%). The highest in E. coli prevalence was from poultry droppings (29.0%), abattoir (14.0%), run off water (14.0%), cattle dung (13.0%), toilet seats (13.0%), dumpsite (10.0%) and the least was door handles (7.0%).

In accordance with the NCCLS standards, standardized confirmatory assays were used to biochemically identify the E. coli isolates. Gram negative bacilli were found in all isolates' Gram stains. Test results for urease, nitrate, and oxidase were positive, as were those for motility, catalase, and indole. The organism was confirmed to be E. coli by the KIA test result, which was positive (yellow/yellow/gas production) (Table 2).

Table 2: Biochemical Identification of E. coli

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

All isolates underwent biofilm assay using the CRA and microtitre plate (96 flat bottom tube) techniques. In different ways, each isolate reacted as a strong, moderate, weak, and non-biofilm forming. Of the 112 E. coli that were isolated, 101 (90.2%) formed biofilm on CRA plates, whereas 11 (9.8%) did not. Table 3 displays these findings, and Plate 1 shows a picture of them. Out of the 112 environmental samples, 97(86.6%) formed biofilm, while 15(13.4%) did not form biofilm on microtitre plates.

According to Table 4, 154 (84.6%) isolates used the microtitre tube method to form biofilm, with an optical density (OD) wavelength range of ≤0,580µm to 2,320µm, ranging from weak to strong formers. In contrast, 28 (15.45) recorded an OD wavelength range of ≥0,580µm, as they were unable to form biofilm and were therefore unable to be picked up by the light.

Discussion
The purpose of this study was to separate and identify E. coli isolates from environmental samples and to find out whether they could form biofilms. One hundred and twelve (112) environmental samples were examined. Every sample and site examined in this investigation had E. coli isolated from it. Ekpoma yielded 44 isolates (39.3%), Iruekpen yielded 22, (19.6%), Opoji yielded 25, (22.3%), and Irrua yielded 21 (18.8%). As a result, the prevalence rate from environmental sources was 112 (28.0%) (Table 1).

A further examination of these findings reveals that the prevalence of environmental samples varied from 7.0% in door knobs to 29.0% in poultry droppings. This demonstrates how common E. coli is in the natural world. Previous studies have also demonstrated that E. coli can be isolated from environmental sources like toilet seats, door handles poultry droppings, cattle dung, abbatoirs, and dumpsites [11-13].

Around the world, the prevalence of E. coli from various specimens varies widely; in Canada, it is 12.8%, while in Iran, it can reach 70.6% [14,15]. While found a low incidence rate of E. coli in another study from Kaduna, observed a high incidence rate in India [16]. According to their debate, E. coli's high prevalence may be due to its resistance to competition from native microbes that develop more quickly.

A biofilm is a surface-attached population of bacteria and their extracellular polymers. Since biofilm formation is linked to pathogenicity, it should be regarded as a significant virulence factor during infections. All isolates were subjected to the biofilm assay utilizing the CRA and the microtitre tube methods, two techniques for biofilm detection, in order to create a baseline for comparison. According to the findings, on CRA plates, 23 (20.5%) had stronger biofilm formers, 32 (28.5%) had intermediate formers, 46 (41.1%) had weak biofilm formers, and 11 (9.8%) had no biofilm formers. This is consistent with other findings by Zhanel et al. (2008), who found that a significant portion of ambient samples included potent biofilm formers.

The CRA approach can be utilized to determine biofilms, according to reports from other investigations. According to Naveen et al. (2014), 46 (38.3%) of the 120 Candida species examined formed biofilm, with 10 (21.7%) being strong and 36 (78.3%) being weak, whereas 74 (61.7%) did not. Additionally, in a related study, Vinitha & Ballal (2011) found that 81 (73%) of 111 Candida spp. generated biofilm utilizing the CRA approach. All of these studies came to the conclusion that the CRA approach is a quick, useful, and trustworthy way to find biofilms in microorganisms. In contrast, the microtitre tube approach yielded 25 (23.3%) findings for strong formers. It was 34 (30.4%) for moderate formers, 38 (33.9%) for weak formers, and 15 (13.4%) for non-formers (Table 3).

Table 3: Rates of Biofilm Formation by E. coli from Environmental Samples

Environmental isolates	Strong formers (%)	Moderate formers (%)	Weak formers (%)	Non-formers (%)	Total isolates (%)
CRA	23(20.5)	32(28.6)	46(41.1)	11(9.8)	112(61.5)
Microtitre Plate	25(23.3)	34(30.4)	38(33.9)	15(13.4)	112(100.0)

Note: environmental samples, a total of 97(86.6%) isolates formed biofilm, 15(13.4%) did not.

Proteus species and P. aeruginosa were 100% biofilm formers in research by using the microtitre tube method, followed by E. coli (77.8%) and S. aureus (80%) [17]. Studies carried out in India revealed similar patterns of biofilm formation (Ponnusamy et al., 2012). However, a study conducted in Egypt found that S. aureus (42.9%) and Klebsiella spp. (44.4%) produced more biofilm than E. coli (31.6%).

Additionally, the calculation of the optical density (OD) value of cells reveals that the greater the OD value, the more adherent the cells are. Despite the fact that the suggested range for OD values is between 540 and 620 µm, calculated an estimated OD wavelength range of 580 µm for this investigation. This study's findings indicate that a total of 15 (13.4%) of the non-formers and 38 (33.9%) of the weak formers had an OD value range of ≥0,580µm, 34 (30.4%) of the moderate formers had an OD value of 1,160µm, and 25 (22.3%) of the strong formers had an OD value of 2,320µm (Table 4). These findings are consistent with those of Mathur et al. (2006), who found that OD values increased during their investigation.

Table 4: Biofilm Formation and Optical Density (OD) Range on Microtitre Tubes

Average OD Range (µm)	Biofilm capacity	Number of environmental isolates
2,320	Strong Formers	25(22.3%)
1,160	Moderate Formers	34(30.4%)
>=0,580	Weak Formers	38(33.9%)
<=0,580	Non-Formers	15(13.4%)

Note: Total of **97(86.6%)** Isolates Formed Biofilm While 15(13.4%) Did Not

Following the demonstration of biofilm formation among isolates, the sensitivity of these two methods was compared, and it was found that both methods had repeatability levels above 80%. The CRA and microtitre tubes, respectively, are used to record this demonstration. When compared to the microtitre tube approach, which had a standardized control, the CRA method's sensitivity to biofilm formation was shown to be the same, despite the lack of a controlled control. Due to the issue of varying individual interpretations of indicated colors, sensitivity may be the same. An internal standard of ISTH, Irrua, Edo State, Nigeria, was employed for this investigation.

Since the microtitre tube method measures the effects of agents against biofilm formation produced on well walls, as opposed to CRA, which simply absorbs the dye's color, it is the "gold standard" method for bacterial cell biofilm formation, according to other reports' studies using a variety of biofilm assay techniques.

Based on factors including sensitivity, specificity, negative predictive value, positive predictive value, and accuracy—all of which were not examined in this study—other researchers recommended that the microtitre tube approach be better and more accurate than the CRA method. False positives were biofilm formers using only the CRA method, whereas true positives were biofilm formers using both the microtitre and CRA methods. Isolates that produced biofilm using the microtitre method but not by CRA were considered false negatives. According to, real negatives are those that were biofilm formers by all means but non-biofilm formers by all means.

The authors came to the conclusion that, although their data conflicted with those of, the microtitre tube method and the tube approach correlate well for powerful biofilm formers. However, the same cannot be true for the CRA method. According to their statistics, which are consistent with those found in this study, they advise that the microtitre approach is more precise and repeatable.

In order to avoid the host's defenses and even antimicrobial drugs, E. coli, like many other bacteria, may build biofilms and endure environmental stress. This work has demonstrated that, provided the growth and survival conditions are favorable, Escherichia coli may be isolated from about every location where microorganisms can grow. This is supported by the fact that every environmental material used in this study included E. coli. Additionally, the isolates demonstrated varying capacity for biofilm formation using both colorimetric and cell adherence (microtitre/optical density assessment) methods.

Conclusion

Environmental samples have revealed the existence of biofilm-forming E. coli. The ideal phenotype—the observable physical characteristics of an organism that are governed by genetics and environmental factors—is expressed by the physiological and genetic engineering programming of bacteria under physio-chemical conditions where control is minimal or nonexistent. As a result, additional research on biofilm-forming bacteria might be conducted to gain a better understanding of them. The goal of future research is to better understand the barrier matrix that bacterial cells in a biofilm state possess as well as the genetic changes that take place in bacterial biofilms [18-23].

Availability of Data and Materials

The authors declare consent for all available data present in this study.

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Competing Interests

The authors declare no conflicts of interest

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