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ISOLATION AND CHARACTERIZATION OF BIOFILM FORMING BACTERIA FROM URINARY TRACT INFECTED PATIENTS

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ABSTRACT: A biofilm is a microbial determined sessile group portrayed by cells that are irreversibly appended to a substratum or interface or to each other. Biofilms are the main cause for increase in virulence and antibiotic resistance of microorganisms. Both gram positive and gram negative pathogens are known to produce biofilms. Due to the production of biofilm, the bacteria adhere to the surface strongly and are protected from any antibiotic agents. The present study is aimed at isolation and characterization of biofilm forming bacteria from UTI samples. Further, N-acylhomoserine lactone (AHL), an autoinducer responsible for the formation of biofilms in gram negative bacteria was characterized. The capacity of the UPEC isolates to form biofilms was analysed using biofilm assay. Further on, N-acylhomoserine lactone was extracted by liquid liquid extraction (LLE) and was estimated colorimetrically and spectroscopically by using fourier transform infrared spectroscopy (FT-IR). The UPEC isolates exhibited strong biofilm forming capacity. The AHL that was extracted showed a characteristic absorbance for C=O bond of lactone ring, and N=H and C-O bond of acyl chain.

INTRODUCTION: Biofilm has been portrayed in numerous frameworks since Van Leeuwenhoek analyzed the plaque on teeth all alone in the seventeenth century and discovered "animalcules". Biofilms are implanted in a network of extracellular polymeric substances which they have created. At long last, they display an adjusted phenotype concerning development rate and quality interpretation. The cells that attach irreversibly to surfaces (*i.e.*, Those not removed by gentle rinsing) will begin cell division, form microcolonies, and produce the extracellular polymers that define a biofilm. These extracellular polymeric substances (EPSs) consist principally of polysaccharides and can be identified minutely and by concoction examination. EPSs give the lattice or structure to the biofilm. They are exceedingly hydrated (98% water) and constantly bound to the fundamental surface.

The structure of the biofilm is not an insignificant homogeneous monolayer of sludge but rather is heterogeneous, both in space and after some time, with "water channels" that permit transport of basic supplements and oxygen to the cells developing inside the biofilm. Biofilms have an affinity to act nearly as filters to capture particles of different sorts, including minerals and host segments, for

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example, fibrin, RBCs, and platelet. Cell communication in bacteria and in some eukaryotic microorganisms occurs in a population-density dependent manner and is based on the production of and response to small pheromone-like biochemical molecules called auto-inducers. Differential gene regulation caused in response to intercellular signaling allows microbes to express a specific behaviour only while growing in communities. This process is termed as quorum sensing to reflect a need to for a certain population to activate the system [1].

N-acylhomoserine Lactone Signaling was first discovered in marine Vibrio species and is called lux type quorum sensing system and is based on production and responses to AHL. The lux type quorum sensing system is archetypal and preeminent mechanism for species specific mechanism in gram negative bacteria. In general, lux type quorum sensing system consists of two components, an autoinducer synthase which synthesizes AHLs (*e.g.*: LuxI) from S-adenosyl methionine, and a transcriptional regulator (*e.g.*: luxR). AHL freely diffuses across the cell membrane because of its small size and lipophilic character. As the population density increases, intracellular AHL binds to the functionally linked (cognate) luxR - like receptor at a sufficient concentration within the cytoplasm to induce differential gene expression. Certain gram negative also use non - AHL systems like quinolone signaling and las and Rhl systems.

The present study is aimed at isolating and characterizing biofilm forming bacteria from patients suffering from urinary infection.

MATERIALS AND METHODS:

Bacterial Source: Four urine samples were collected from the patients suffering with UTI and were being treated at Bhagwan Mahaveer Jain Hospital, Bengaluru. Samples were collected in sterile and clean urinary containers and cultivated for identification.

Colony Development of Urine Samples: All samples were inoculated by streaking on MacConkey agar and incubated at 37 °C for 24 hours. Bright pink coloured colonies were observed. Five different well isolated distinct colonies were observed. Pure cultures of these colonies were obtained by sub culturing on MacConkey by quadrant streaking and slant streaking. Each isolate was given a number before its identification and these numbers were maintained throughout.

Biochemical Identification of Isolates: Sub cultures of isolates were further identified and characterized by various biochemical tests namely indole, MR-VP, citrate utilization, sugar fermentation test [2]. The morphology was identified using Gram's staining method by Hans Christian Gram [3].

Primary Attachment Assay: Primary attachment assay was carried out according to the protocol explained by Rohollah Taghadosi *et al*., [4]. One loopful of identified cultures was inoculated in TSB media and were incubated for 24 hours at 37°C. Overnight grown cultures were diluted into fresh TSB media containing 1% glucose in the ratio 1:100. The optical density of the cultures was adjusted to an optical density of 0.5 at 600 nm. This 1×10^8 CFU/ml suspension of culture was diluted to 10^3 CFU/ml with TSB media. 100 µl aliquots of the dilutions were added per well in a 96 wells microtiter plate. 100 µl of uninoculated medium were used as negative control. Microtiter plate containing aliquots were incubated for 30 minutes at 37 °C. After 30 minutes the cultures were removed from the plates and were rinsed with sterile 0.1 M phosphate buffer saline (PBS). 150 µl of TSB was then added to each well and optical density was read at 600 nm. Triplets were maintained for each culture.

Biofilm Formation Assay by Microtiter Plate Method: Quantification of biofilm formation in each UPEC isolate was assayed by Microtiter plate method as described by Stepanović S *et al*., with some modifications [5]. *E. coli* from UPEC isolate was inoculated into 2 ml of sterile TSB medium containing 1% w/v glucose for optimization of biofilm production. Medium containing *E. coli* was incubated to achieve an optical density of one at OD600. 1.5×10^8 CFU/ml suspension was further diluted to achieve dilution of~10^6 CFU/ml. To the wells of flat-bottom 96-well microtiter plate, 100 µL of each of the dilutions of prepared bacterial suspension were added.

For negative control, 100 µL of uninoculated medium were used. Bacteria were grown and adhered to the wells of microtiter plate without agitation for 24 hours at 37 °C. After the incubation, cells which were non- adherent were aseptically aspirated and were washed using water. 10 µL of PBS (pH 7.2) was then added to the wells. PBS was replaced with 150 µL of methanol and wells were incubated for 20 minutes at room temperature. After incubation, methanol was removed and 200 µL of 1% w/v crystal violet was added to each of the well. The wells were then washed with sterile deionized water gently and plates were kept for air drying at room temperature. Once the plates were dried 200 µL of 33% v/v glacial acetic acid were added to the wells and the OD was measured at 490 nm in a microtiter plate reader. All the isolates were performed in triplets.

Extraction of AHL from UPEC Isolates: N-Acyl Homoserine Lactone was extracted from UPEC isolates by the method as described by Dietrich JA *et al*., [6]. Loopful of bacterial isolates were inoculated in Muller Hinton Broth and incubated for 24 hours at 37 °C. From the overnight grown culture, 1.5 ml of the culture was aseptically transferred to the sterile centrifuge tubes and was centrifuged at 10,000 rpm for 15 minutes. After centrifugation, the culture supernatant was retained for AHL extraction by Liquid Liquid extraction method. Supernatant was mixed with equal amount of ethyl acetate and was shaken for about 10 minutes. Mixture was allowed to stand for 10 minutes undisturbed. To form upper organic and lower aqueous layer. The upper layer was aspirated by a micro pipette and from the lower portion, extraction was repeated two more times by dissolving in ethyl acetate. The upper portion of each extracted samples were collected and dried in an oven at 40 °C. After drying, the powdered extract was dissolved in ethyl acetate.

Detection of AHL by Colorimetric Method: For detection of AHL, High throughput detection method of quorum sensing molecule by Yang YH *et al*., was performed [7]. 40 µL of AHL extract of each of the sample were added into the wells of 96-well polystyrene flat-bottom microtiter plates. Mixture of 2 M hydroxyl amine and 3.5 M NaOH in the ration 1:1 was prepared and 50 µL of this mixture was aliquoted to the wells containing samples. 50 µL of 1:1 mixture of 10% ferric chloride in 4 M HCl and 95% ethanol was added to the same wells containing sample. The OD was measured at 520 nm.

Determination of AHL Functional Groups: AHL extracted were analyzed for their lactone functional groups as described by Houshang Shikh-Bardsiri *et al*., by Bruker-alpha FT-IR [8]. A drop of Extracted AHL was placed in between two KBr plate and was subjected to IR.

RESULTS AND DISCUSSION:

Colony Development of Urine Samples: Four urine samples infected with Uropathogenic *Echerchia coli* were collected from Bhagwan Mahaveer Jain Hospital, Bengaluru.

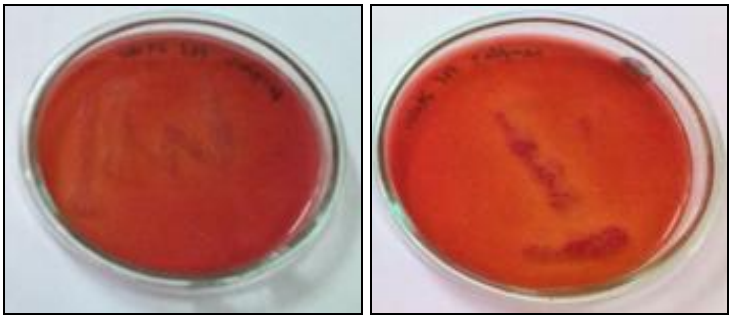
MacConkey media is a differential and selective culture media which is used for the growth of Gram negative and enteric bacteria. Differentiation between the bacteria is based on lactose fermentation. After 24 hours of incubation, colonies were grown on MacConkey agar plates which were subjected to characteristics and morphology. **Fig. 1** illustrates well isolated pink to red coloured colonies of lactose fermenting bacteria appearing on the plates and was surrounded by bile zone of precipitation.

Control plate was incubated without any organisms and it represents the MacConkey agar without any growth. Light pink colonies were obtained from sample 1 and there was no change in colour of the media. Pink colonies were obtained from sample 2. Faded pink colonies were obtained from sample 3 and the media colour changed to yellow around the colonies. Two colonies were obtained from sample 4, one was light pink coloured and the other was faded pink colony and the media colour changed to yellow.

Subculture of Isolated Colonies: Importance of this step is to isolate pure colonies of bacteria. Each of the well isolated colony from the main culture were aseptically transferred on to different MacConkey agar plate to obtain pure cultures. Out of all the four cultures, Sample 4 had two different types of colonies, which were streaked separately. There were total five pure cultures that were obtained at the end. There was one subculture obtained from samples 1, 2 and 3, and two

subcultures were obtained from sample 4. The characteristic features of the colonies on solid agar media are then noted as shown in **Table 1**. These include: Shape- circular, irregular or rhizoid; Size- small, medium or large; Elevation-elevated, convex, concave or umbilicate; Surface- smooth, wavy, rough, granular or mucoid; Edges- entire, undulate, crenate, fimbriate or curled; Colour- yellow, green, pink *etc*; Structure- opaque, translucent or transparent; Degree of growth- scanty, moderate or profuse.

CONTROL **SAMPLE 1** **SAMPLE 2**



SAMPLE 3 **SAMPLES 4**

FIG. 1: COLONIES FROM DIFFERENT UTI SAMPLES

SUBCULTURE 1 **SUBCULTURE 2** **SUBCULTURE 3**

SUBCULTURE 4 (A) **SUBCULTURE 4 (B)**

FIG. 2: SUBCULTURES OF THE UTI SAMPLES

TABLE 1: COLONY CHARACTERISTICS OF MICRORGANISMS ISOLATED FROM URINE SAMPLES

Isolate No.	Shape	Elevation	Margin	Colour
1	Circular	Elevated	Undulated	Pink
2	Circular	Elevated	Smooth	Pink
3	Circular	Flat	Smooth	Pink
4(a)	Circular	Elevated	Undulated	Pink
4(b)	Circular	Flat	Smooth	Pink

Biochemical Identification of Isolates: The identification of bacteria is a careful and systemic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture. Multiple tests were performed to provide the fermentation abilities, presence of certain enzymes and certain biochemical reactions. Qualitative observations were made based on the tests, which were compared as the bacteria identification key to aid identification process.

Indole Test: Indole test is based on the presence of an enzyme called tryptophanase which determines the ability of bacteria to split amino acid Tryptophan. As a result of this, three end products namely indole, pyruvate and ammonium are produced. Indole production is determined by Kovac's Reagent which contains 4-p diethylamino benzaldehyde which reacts with indole to produce a red coloured compound. All the samples showed positive result for Indole [9].

Methyl Red Test: Methyl red test is based on the ability of bacteria to perform mixed acid fermentation when supplied with glucose. Enteric bacteria metabolize glucose to produce pyruvic acid. Some enteric bacteria then use mixed acid pathway to metabolize pyruvic acid to other acidic products such as acetic, lactic and succinic acid. Large amount of acid production reduces the pH to 4 or below. This lower pH is then determined by methyl red pH indicator. Acidic pH (below 4.4) gives red colour and basic pH (above 7) gives yellow colour after the addition of pH indicator. All the samples showed positive result for methyl red test.

Voges Proskauer Test: In some organisms, the end products of glucose metabolism, pyruvic acid, is further metabolized by using Butylene glucol pathway to produce neutral end products such as acetoin and 2, 3 butanediol. Presence of acetoin is determined using Barrit's Reagent A (40% KOH) and Barrit's Reagent B (5% solution of alpha naphthol). Acetoin in presence of oxygen and Barrit's Reagent is oxidized to diacetyl, where alpha naphthol acts as catalyst. Diacetyl then reacts with guanidine components of peptone to produce a cherry red colour. All the samples showed negative result for Voges Proskauer test [9].

Citrate Utilization Test: This test determines the ability of microorganisms to utilize citrate. Some bacteria have capability to convert salts of organic acids such as sodium citrate to alkaline carbonates. Sodium citrate is one the important metabolite of Krebs cycle. Certain bacteria use citrate as the sole carbon source. Citrate is converted to oxaloacetic acid by citrate lyase and oxaloacetate decarboxylase activity will convert oxaloacetate to pyruvate with the release of carbon dioxide. The other products of the reaction are acetate, lactic acid, formic acid *etc*. The carbon dioxide reacts with sodium and water to form sodium carbonate. Sample 2, Sample 3 and Sample 4(b) were citrate negative whereas Sample 1 and Sample 4(a) showed positive result for citrate test. For further confirmation samples were tested for Metallic Sheen on EMB plates [9].

Sugar Fermentation Test: Sugar fermentation test is performed to check for the ability of a microorganism to ferment the carbohydrate which is indicated by presence of gas. Basal medium containing 1% of single carbohydrate source with an inverted Durham's tube is used for the test. Production of hydrogen or carbon dioxide due to fermentation process forms air bubble in Durham's tube, which indicates positive result for the test. All the samples showed positive result for sugar fermentation test [2].

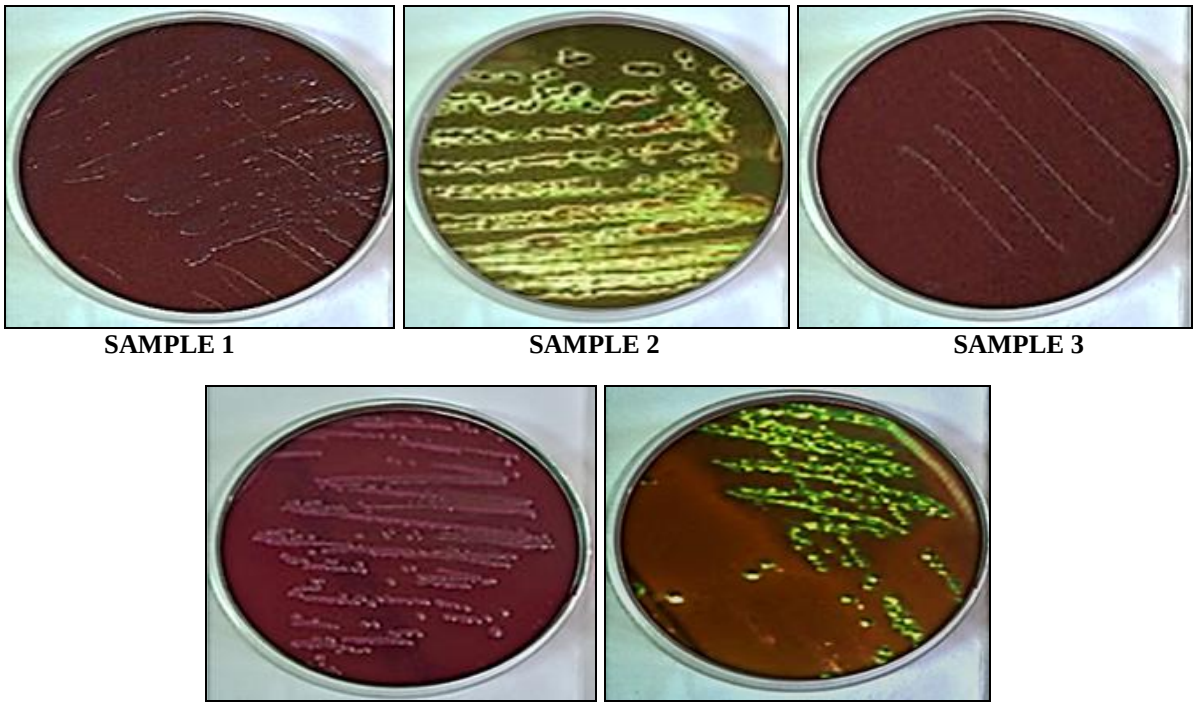
Gram Staining: This is a differential staining process developed by Hans Christian Gram 1884, which separates microorganisms based on their cell wall composition. Microorganisms which stains purple are gram positive bacteria because they have thick layer of peptidoglycan (90% of cell wall) which takes up the primary stain (crystal violet).

Whereas Gram negative bacteria has thin layer of peptidoglycan (10% of cell wall) and high lipid content and are stained pink by counterstain [3]. Five UPEC isolates were subjected to above biochemical tests for identification of microorganisms. Following results were obtained as tabulated in **Table 2**.

TABLE 2: BIOCHEMICAL CHARACTERISTICS, GRAM STAINING AND MORPHOLOGY OF UPEC ISOLATES

Isolate no.	Indole test	MR test	VP test	Citrate utilization test	Sugar fermentation test			Morphology	Gram Stain
					glucose	lactose	Sucrose		
1	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Rods	Negative
2	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Rods	Negative
3	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Rods	Negative
4(a)	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Rods	Negative
4(b)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Rods	Negative

Metallic Sheen on EMB Agar: Due to positive results for citrate utilization test, EMB agar test was performed which differentiate between Enterobacter and *E. coli.* Most of the strains of *E. coli* colonies develop a characteristic green sheen when cultured on EMB agar plate. Rapid fermentation of lactose and production of strong acids reduces the pH of the EMB agar and forms a green metallic sheen with *E. coli*. Lactose non-fermenters are either colorless or light lavender. EMB media assists visual distinction of *E. coli* from other pathogenic enteric bacteria as illustrated in **Fig. 3**. Metallic sheen were obtained for sample 2 and sample 4(b) whereas for Sample 1, Sample 3 and Sample 4(a) there were no development of metallic sheen and hence negative for EMB test. Hence for further analysis, Sample 2 and Sample 4(b) were used.



SAMPLE 1 **SAMPLE 2** **SAMPLE 3**

SAMPLE 4A **SAMPLE 4B**

FIG. 3: COLONIES ON EMB AGAR

Primary Attachment Assay: The result of primary attachment assay is depicted in **Fig. 4**, which indicates the attachment of bacteria to the microtiter plates. This test was performed to ensure the attachment of the bacteria to the wells of the microtiter plate. 10^8 showed maximum biofilm forming capacity due to more number of colonies, 10^7 showed comparatively lesser biofilm forming capacity due to reduced number of colonies. As dilution increases, the biofilm forming capacity reduces.

Biofilm Formation Assay: Illustrated below in the **Fig. 5** is the results obtained from biofilm formation assay which included incubation period of 24 hours. The isolates exhibited high biofilm forming capacity. Large incubation period helps in more attachment of bacteria. According to this assay the biofilm formation of the bacteria depends on the number of bacteria remaining in the well. The two isolates displayed a highly positive biofilm forming strain. These highly positive biofilm forming property of bacteria helps them to persist on the wall of microtiter well or on the wall of urogenital tract causing virulence [10].

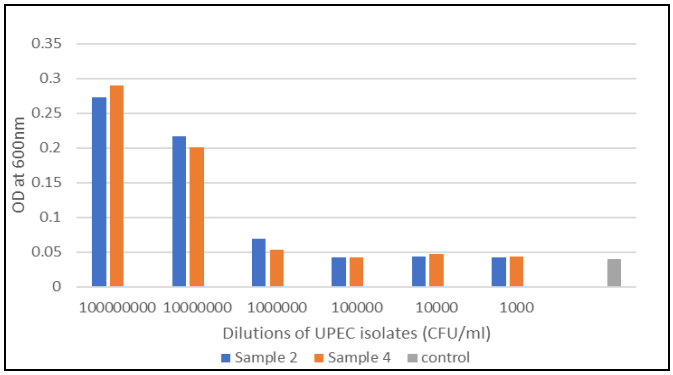


FIG. 4: PRIMARY ATTACHMENT ASSAY

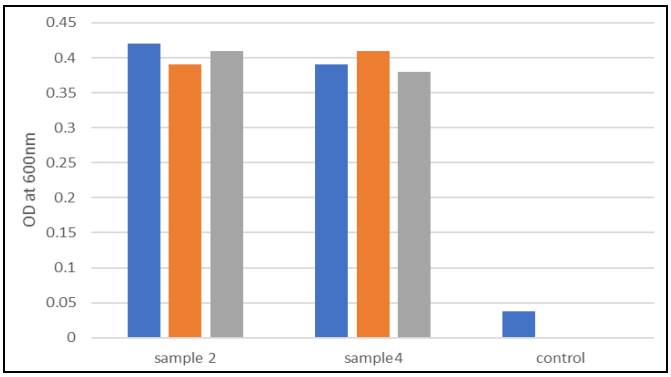


FIG. 5: CAPACITY OF BIOFILM FORMATION BY DIFFERENT ISOLATES, SERIES 1, 2 AND 3 REPRESENTS THREE DIFFERENT DUPLICATES OF A SAMPLE

Estimation of AHL by Colorimetry Method: Calibration Curve: Standard curve for AHL was obtained using standard lactone compound phthalic anhydride [7]. Under alkaline conditions AHLs are rapidly inactivated by pH-dependent lactonolysis, in which the homoserine lactone ring is hydrolyzed to open ring form corresponding to N-acylhomoserine. This reaction can be reversed by acidification; therefore, we monitored pH throughout the experiment to verify that an acidic pH was maintained.

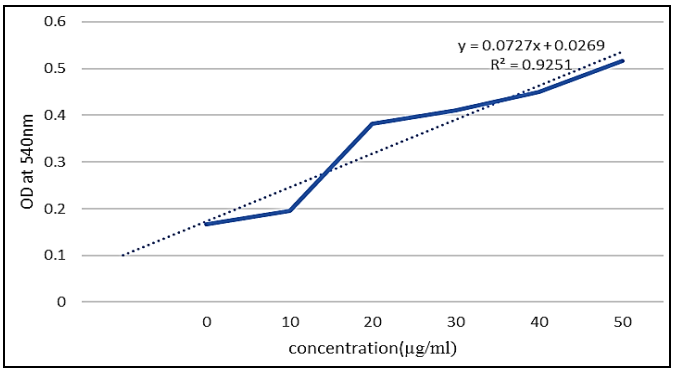


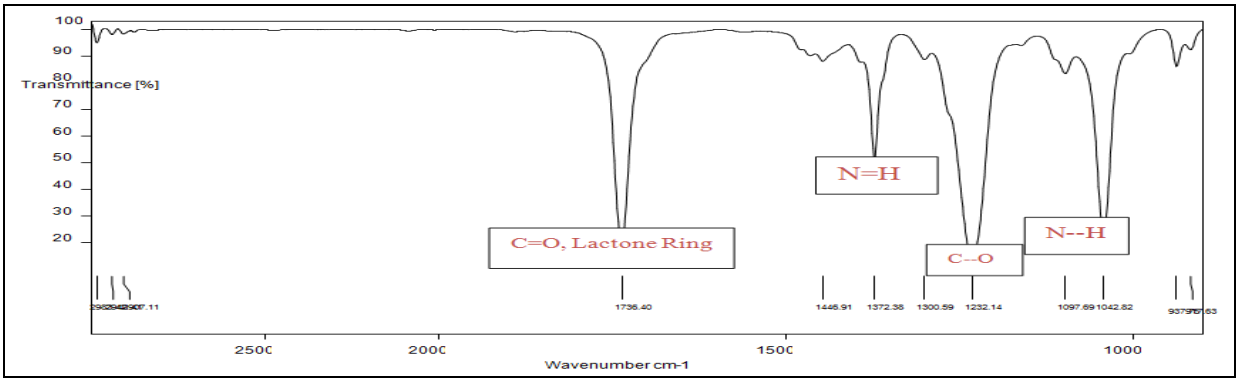
FIG. 6: STANDARD CURVE FOR ESTIMATION OF AHL

From the standard graph of phthalic anhydride (y = 0.0727x + 0.0269, R^2 = 0.9251), total amount of AHL in sample 2 was found to be 2.325 ± 0.02µg/ml and that of sample 4 was found to be 2.70 ± 0.02µg/ml. Phthalic anhydride is a lactone compound and shows a similar reaction under alkaline conditions and hence was taken as a standard.

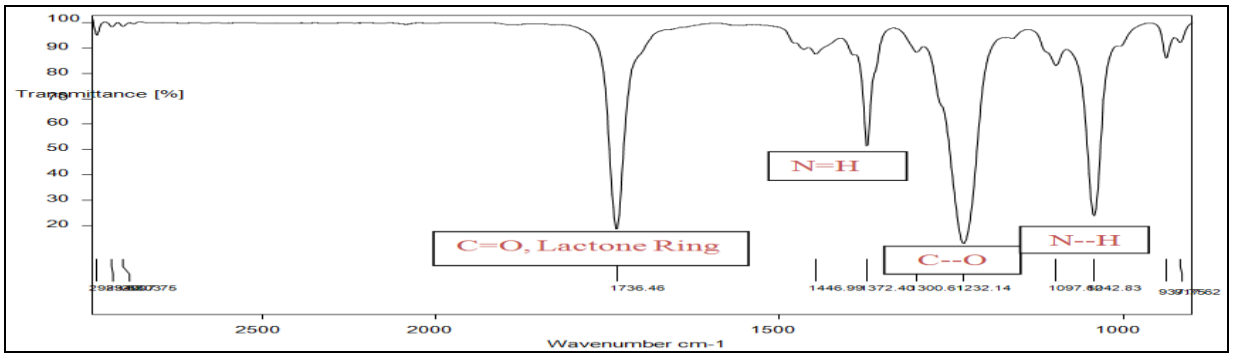
TABLE 3: ABSORBANCE SHOWED BY AHL AT 540 nm

Sample	OD at 540 nm	
Sample 2	0.217	0.219
Sample 4	0.269	0.271

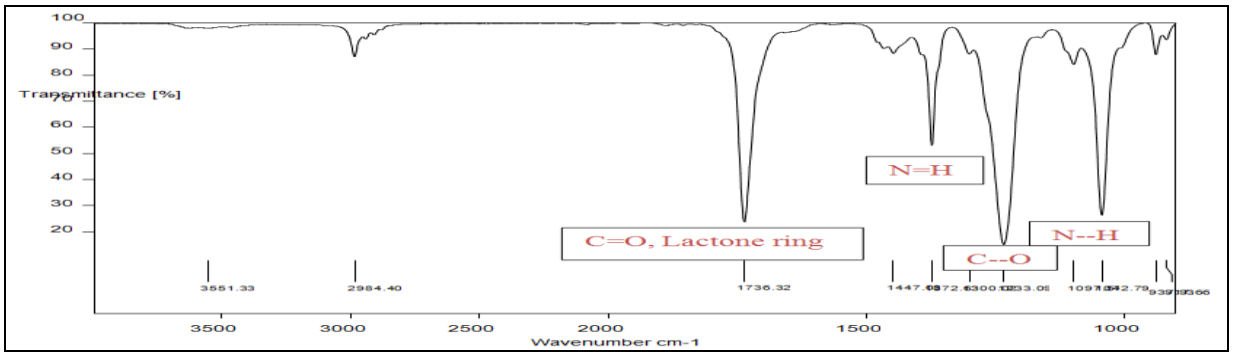
Determination of AHL Functional Group: Fourier Transform Infra-Red Spectrophotometer of the extracted AHL sample, along with standard molecule was performed to identify the organic solvent present. It relies on the principle that most molecules absorb light in infrared region (4000 - 600 cm^{-1}) of electromagnetic spectrum. The absorption peaks correspond directly to the bond present in the sample to be identified. For determination of AHL, strong peaks in the range 1750 - 1735 and 1300 - 1000 cm^{-1} correspond to the C=O bond of lactone ring and the C-O bond, respectively should be obtained [4]. Following are the FT-IR plots for standard and two unknown samples which were extracted from the two bacterial samples. Phthalic anhydride was taken as a standard and peaks were obtained at 1739.40, 1372.99, 1222.14 and 1042.82 cm^{-1} and these peaks correspond to C=O of lactone ring, N=H bond, C-O and N-H respectively. Similar peaks were obtained for unknown samples 2 and 4.



SAMPLE 1



SAMPLE 2



SAMPLE 4B

FIG. 7: FT-IR ANALYSIS

CONCLUSION: It has been observed that the UPEC isolates have a strong biofilm forming abilities. The key component that is responsible for biofilm formation is N-acylhomoserine lactone in gram negative bacteria. The UPEC isolates with biofilm forming ability was subjected to N-acyl homoserine lactone quantification assay. AHL was extracted by liquid-liquid extraction. Extracted AHL was quantified using standard curve of phthalic anhydride. Qualitative estimation of AHL was done by FT-IR. Corresponding peaks were obtained for lactone ring when FT-IR was performed. Further research can be conducted to study the genetics of AHL in *E. coli* and also its importance in the biofilm forming capacity of an organism.

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CONFLICT OF INTEREST: No potential conflict of interest was reported by the authors.

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