IJPSR (2014), Vol. 5, Issue 7 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 20 January, 2014; received in revised form, 05 May, 2014; accepted, 03 June, 2014; published 01 July, 2014

MULTI-DRUG RESISTANT PATTERNS OF BIOFILM FORMING *AEROMONAS HYDROPHILA* FROM URINE SAMPLES

S. Thenmozhi*, P. Rajeswari, B.T. Suresh Kumar, V. Saipriyanga and M. Kalpana

Department of Microbiology, Vivekanandha College of Arts and Sciences for Women (Autonomous) Tamil Nadu, India

Keywords:

Urine, *Aeromonas hydrophila*, Slime, Biofilm, Multi-drug Resistance, MAR Index

Correspondence to Author:

S. Thenmozhi

Department of Microbiology, Vivekanandha College of Arts and Sciences for Women (Autonomous), Elayampalayam- 637205, Tiruchengode, Namakkal District, Tamilnadu, India

E-mail: stmmicro@gmail.com

ABSTRACT: Biofilm are a matrix of microorganisms which are adhered to and colonized a surface. When formed they are very difficult to remove and act as a source of contamination in processing environments. As bacteria in biofilm exhibit enhanced resistance to antibiotics and clearance by the host immune system, the resistance of enteropathogenic bacteria to commonly prescribed antibiotics is increasing both in developing as well as in developed countries. Resistances have emerged even to newer, more potent antimicrobial agents. This study was under taken to investigate the presence of multidrug resistance producing biofilm forming *Aeromonas hydrophila* in human clinical samples. A total of 150 urine samples were collected from private hospital in Tiruchengode during the period of six month. Among these only 75 isolates were found to be positive for *Aeromonas hydrophila*. The Starch-Ampicillin agar were used as a selective presumptive isolation medium for the isolation of bacterial isolates and confirmed as *Aeromonas hydrophila* were determined by using standard biochemical analysis according to Bergey's manual of systematic Bacteriology. Slime producing isolates were studied on Congo Red Agar (CRA) plate method and the biofilm were determined in tube method. Multi-drug resistance patterence and MDR index were carried out according to the criteria of national committee for clinical laboratory standards. Infection due to bacterial pathogen with such virulent factors (biofilm) act as a one of the source for multi-drug resistance producing isolates among the microbial population. *Aeromonas hydrophila* has received particular attention because of its association with human infection. So that, in this present study the slime and biofilm forming isolates was detected and studied their multi-drug resistance patterns. Urine samples were collected from private hospital in Tiruchengode was found to contain very diverse populations of biofilm forming *Aeromonas hydrophila*.

INTRODUCTION: In recent years, there has been an increasing number of reports on diverse *Aeromonas* sp. associated infections, including endocarditis, gastroenteritis, hemolytic-uremic syndrome, meningitis, pneumonia, septicemia, urinary tract infections, Wound infections, etc. Urinary tract ifection (UTI) remains the common infections diagnosed in outpatients as well as in hospitalized patients. Worldwide data show that there is an increasing resistance among urinary tract pathogens to conventional drugs.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(7).2908-18

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(7).2908-18

Clinical and epidemiologic evidence indicates that *Aeromonas* spp. are enteropathogenic despite the fact that very few well documented outbreaks have been reported [1].

Aeromonas species are ubiquitous microorganisms found in both aquatic and environmental habitats. They are Gram negative, short rod shape, oxidase and catalase positive, motile, facultative anaerobes, resistant to 0/129 vibriostatic agent and non-spore forming. Nineteen species of the genus have been identified till date [2, 3].

Virulence factors such as aerolysin, haemolysin, cytosine, enterotoxin, proteolytic activity, lipolytic activity, gelatinase, slime production and antimicrobial peptides have been identified in *A. hydrophila* . These virulence factors are used as survival means, self defense mechanism and establishment of pathogenicity [4]. In a research in 1995,some researchers stated that virulence factors are determinant of bacterial pathogenicity .These are mostly found in bacteria including *Aeromonas* spp.

Aeromonads have been attributed to human infections like *gastroenteritis*, urinary tract infection, septicemia and wound infections. Protease, Aerolysin, Hemolysin, Enterotoxins, Lipases, Gelatinase and Biofilm formation as virulence factors in *Aeromonas* spp.[5]. Biofilm is an irreversible growth of aggregated bacterial micro-colonies on surfaces embedded in extracellular polysaccharide matrix. Biofilm formation results into resistance of bacteria to conventional antibiotics and persistent infections [6].

Slime: Slime is another type of virulence factor, which is a viscous glycoconjugate material produced by most of the Gram negative bacteria. It is also helpful in the formation of biofilm.The slime highly significant to the pathogenesis. It appears to inhibit the neutrophil, Chemotaxis, Phagocytosis and antimicrobial drugs [7].

Biofilm forms when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor them to all kinds of material – such as metals, plastics, soil particles, medical implant materials, and tissue.

Biofilms: The discovery of microorganisms, 1684, is usually ascribed to Antoni van Leeuwenhoek, who was the first person to publish microscopic observations of bacteria.

Although the most common mode of growth for microorganisms on earth is in surface associated communities, the first reported findings of microorganisms "attached in layers" were not made until the 1940s. During the 1960s and 70s the research on "microbial slimes" accelerated but the term "biofilm" was not unanimous formulated until 1984 [8]. Various definitions of the term biofilm have been proposed over the years. According to the omniscient encyclopaedia Wikipedia a biofilm is "a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface" (http://en.wikipedia.org, 20090205).

Biofilm formation and development: Biofilm formation and development is a fascinatingly intricate process, involving altered genetic genotype expression, physiology and signal molecule induced communication. Biofilms can form on all types of surfaces, biotic or abiotic, in most moist environments. Several distinct steps essential in the biofilm formation process have been identified and a simplified sketch of the most crucial ones can be seen in **Figure 1**.

Surfaces in aquatic environments generally attain a conditioning film of adsorbed inorganic solutes and organic molecules **(Figure 1.1).** Bacteria move towards the surface by chemotaxis or Brownian motion, resulting in a temporary bacteria-surface association **(Figure 1.2)** mediated by non-specific interactive forces such as Van der Waals forces, electrostatic forces, hydrogen bonding, and Brownian motion forces [9]. At the surface, production of extracellular polymeric substances will firmly anchor the cells to the surface. This state is commonly referred to as irreversible attachment **(Figure 1.3),** truly irreversible only in the absence of physical or chemical stress. Synthesis of exopolysaccharides which form complexes with the surface material and/or secretion of specific protein adhesins that mediate molecular binding are known mechanisms for irreversible attachment.

A large group of such proteinaceous adhesins are the b-sheet-rich, water insoluble amyloid fibrils found in 5-40% of the strains present in both freshwater and wastewater treatment biofilms. During the initial attachment various short range forces are involved, including covalent, hydrogen and ionic bonding as well as hydrophobic interactions. The initially adhered cells rarely come in direct contact with the surface because of repulsive electrostatic forces; instead the secreted polymers link the cells to the surface substratum. The shift from reversible to irreversible attachment is relatively rapid. Various studies report firm attachment within a few minutes or less. Once anchored at the surface, cell division and recruitment of planktonic bacteria results in growth and development of the biofilm community, i.e. maturation **(Figure 1.4).**

Surface attached bacterial cells use the nutrients in the conditioning film and the aqueous bulk to grow and produce more EPS resulting in the formation of microcolonies. Eventually the microcolonies expand to form a layer covering the surface. During biofilm growth a differentiation of the gene expression pattern can be seen compared to planktonic cells. The production of surface appendages involved in bacterial motility is down-regulated due to cell immobility in the biofilm matrix while production of EPS and membrane transport proteins such as porins is up-regulated [10]. The up- and down-regulation of genes is mainly dependent on population density and is controlled by a signal molecule driven communication system known as quorum sensing.

Mature bacterial biofilms are dynamic, spatially and temporally heterogeneous communities which can adopt various architectures depending on the characteristics of the surrounding environment (nutrient availability, pH, temperature, shear forces, osmolarity) as well as the composition of the microbial consortia. Complex structures such as mushroom-like towers surrounded by highly permeable water channels, facilitating the transport of nutrient and oxygen to the interior of the biofilms, are commonly observed.

The biofilm development process is fairly slow; several days are often required to reach structural maturity. A mature biofilm is a vibrant construction, with an advanced organisation which continuously adapts itself to the surroundings, meaning that under adverse conditions bacteria may leave their sheltered existence within the biofilm community in the search for a new, more favourable habitat to settle down in. This step is known as detachment **(Figure 1.5).**

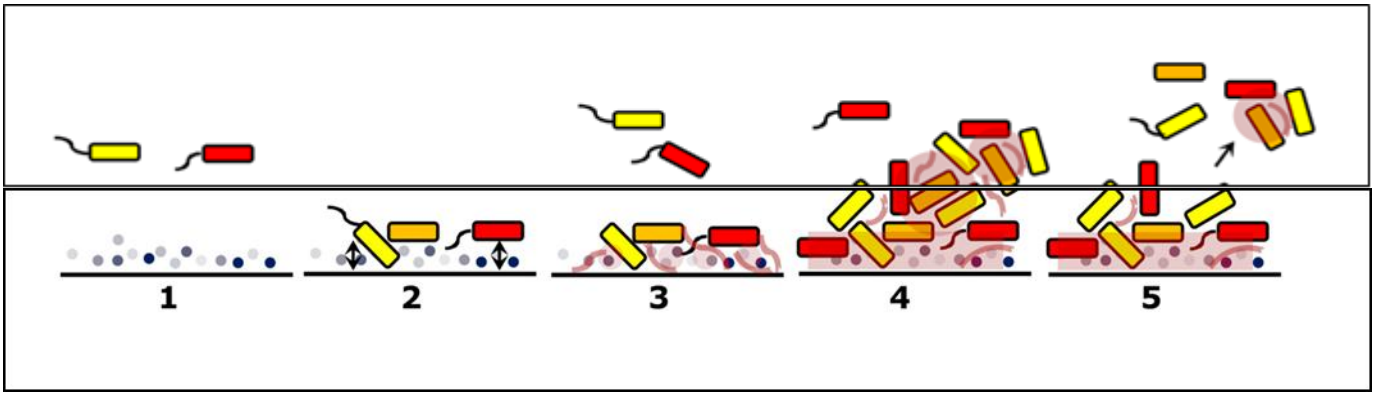

FIG. 1: SCHEMATIC REPRESENTATION OF THE STEPS INVOLVED IN BIOFILM FORMATION. 1.1. FORMATION OF CONDITIONING FILM ON THE SURFACE, 1.2. INITIAL ADHERENCE OF BACTERIAL CELLS, 1.3. IRREVERSIBLE ATTACHMENT OF BACTERIA, 1.4. MATURATION OF THE BIOFILM, 1.5. DETACHMENT.

Multi-drug resistance by biofilm: Many antibiotics were intensively used worldwide to control bacterial infectious diseases, but on using these antibiotics the drug resistance patterns had been developed within the pathogenic communities. Therefore, the production of alternatives to the ordinary used antibiotics gained big importance in many countries. MDR (Multi Drug Resistance) is a phenotype increasingly associated with many pathogens.

MDR can be caused by simultaneous presence of multiple individual resistance mechanisms, each of which can be either plasmid- or chromosome-mediated. In a typical example, and R plasmid, which is often transferable or conjugative, causes MDR because it contains multiple resistance genes on a single molecule of DNA. Furthermore, the so-called resistance island, often on a chromosome, may contain a cluster of multiple resistance genes. Resistance genes are often also co-present with mobile genetic elements, e.g., transposons and integrons, and in this manner they move as a block between molecules of DNA, for example among different R plasmids and between plasmid and chromosome.

Biofilms exhibit an inherent resistance to all classes of antimicrobial agents such as antibiotics, disinfectants and germicides. EPS, which encases the biofilm, functions as a diffusional barrier to antimicrobial agents. The nutrient availability gradually, decreases in the depth of biofilm as the EPS interferes with flow of nutrients, just the way it does with the diffusion of antibiotics. The result is the existence of slow growing or starvation state of bacteria in biofilm [11].

Most antimicrobials require at least some degree of cellular activity to be effective; since their mechanism of action usually relies on disrupting different microbial metabolic processes. So, existence in biofilm of bacterial population in a wide variety of metabolic states and the fact that slow growing and non-growing cells are less susceptible to antibiotics in comparison to actively growing cells, contributes significantly to resistance of biofilm bacteria to antibiotics [12].

Biofilm bacteria in general exhibit higher levels of resistance to all classes of antibiotics. In comparison to their non-attached, individual planktonic counterparts, biofilm bacteria are in the range of 10-2000 times more resistant. Multiple mechanisms are involved in resistance of bacteria in biofilm to antimicrobial agents.

First, depending of the type of biofilm and the poor penetration of biofilm by antimicrobial agents as the EPS which constitute the biofilm retard the diffusion of the antibiotics and the drugs cannot penetrate the full depths of the biofilm matrix [13].

Rate of penetration also varies with the nature of the drug and structure of the biofilm. Antibiotic ciprofloxacin required 21 minutes versus 4 sec to reach a surface when the surface was coated with a *P. aeruginosa* biofilm or when no biofilm was present. Comparative analysis of susceptibility to antibiotic tobramycin revealed that biofilm cells were 15 times more resistant to the drug than their isogenic, planktonic counterparts [14].

As the antimicrobial resistance of biofilm is higher, use of antibiotic at recommended dose is often unable to eradicate biofilm infection. Challenging biofilm with such sub-lethal dose often leads to partial disruption of biofilm, facilitating repopulation and formation of biofilm at newer locations 22. As bacteria from a biofilm have enhanced potential to form new biofilm in comparison to their isogenic, planktonic counterparts 20, the eradication of the newer biofilms thus formed may be more difficult. The objective of this study was to determine the multi-drug resistance patterns of biofilm forming *Aeromonas hydrophila* from urine samples was investigated.

MATERIALS AND METHODS:

Study area: In this study, the commonly infected person samples (Urine) were collected from private hospital in Tiruchengode. Urine acts as a natural medium for the growth and isolation of large number of pathogenic and non-pathogenic microorganisms. This microbial population produces different types of virulence factor (enzymes) when it comes under the unfavourable (or) infectious stage.

Finally it will leads to the multi-drug resistance specifically in the recent years the biofilm producers was highly resistant to multiple antibiotics particularly for third and fourth generation antibiotics So that, in this study the importance is given to determine the multi-drug resistance patterns of biofilm forming *Aeromonas hydrophila* from urine samples.

This study showed that the biofilm is a one of the highly attention needed virulence factor in clinical and environmental aspects.

Sampling methods: A total of 150 urine samples were collected from private hospital in Tiruchengode during the period of six months (January 2013 to June 2013). Urine specimens were collected carefully without any urethral contaminations. A wide mouth screw cap bottle was used for urine sample collection. Overfilling was avoided. Clean catch mid-stream urine used for the routine microbiological purpose collected samples were brought to the laboratory with the aid of ice pack.

Isolation and Preservation: The urine samples were directly streaked in to different media such as Nutrient agar, MacConkey agar and selective media such as Starch Ampicillin Agar (SAA) (Hi-Media, Mumbai, India) and incubated at 37°C for 24-48 h for the isolation of organisms [15]. The selected colony was once again streaked on the selective media for the pure-culture isolation. Colonies of presumptive isolates were stained and then identified as *Aeromonas* spp. based on morphology, motility, catalase and oxidase test. Then the identified colonies were subsequently maintained in Brain Heart Infusion Agar slants (BHIA) at 4ºC.

Identification of bacteria: The experimental isolates of *Aeromonas* spp. was isolated from above method were subjected to study their morphological and biochemical tests as recommended by using standard biochemical tests. Further, it was tested in Kaper's multitest medium [16].

Determination of Virulence character:

1. **Slime production assay (Congo Red Agar Method):** Colony morphology and phenotypic change of slime producing isolates were studied on CRA, which requires the use of a specially prepared solid medium, Brain Heart Infusion Agar (BHIA) supplemented with 5% sucrose and Congo red dye. Congo red was prepared (0.086mg/l) as concentrated aqueous solution and autoclaved separately and added to the sterilized BHIA at 55˚C. The isolates were streaked to a length of 1.5cm on Congo red agar plate and incubated at 37˚C for 24hrs and subsequently kept at room temperature. Black colonies were considered to be positive variants, while red colonies were considered to be negative [17].

2. **Biofilm formation assay - Standard Tube Method (STM):** The method used for quantitative analysis of the isolates for biofilm production. Briefly, a colony of each *Aeromonas* spp was inoculated into 10 mL of Trypticase Soy Broth (TSB) supplemented with 1 % w/v of glucose. Tubes were then incubated at 30°C for 18 – 24 hours [18]. Tube contents (sessile cells) were decanted and washed thrice with 1x phosphate buffer saline (PBS). The tubes were drained and dried by inversion. 1 % w/v of Safranin was used to stain the dried tubes for 5 minutes [19]. Presence of adherent stained film was taken as positive result. However, adherent stained film at liquid and air interface was disregarded as positive result.

3. **Multi-drug resistance patterns of biofilm isolates:** Antibiotic susceptibility of *A. hydrophila* isolates and indicator bacteria was carried out using Mueller-Hinton agar (MHA, Merck) following manufacturer's instruction by agar disc diffusion method [20]. Each isolate was aseptically streaked on MHA using sterile swab. The following antibiotics discs were then placed on the surface of the solidified Agar and allowed to diffuse into the agar for 10 -15 minutes before incubating at 30°C for 18 - 24 hours.

   A total of 12 chemotherapeutic agents (Oxoid), were used Azithromycin (At) (15mcg), Amikacin (Ak) **(**30mcg), Gentamicin (G) (10mcg), Ciprofloxacin (Cf) (5mcg), Cephadroxil (Cq) (30mcg), Cefuroxime (Cu) (30mcg), Roxithromycin (Ro) (10mcg), Ampicillin/ cloxacillin (Ax) (10mcg) Cephotaxime (Ce) (30mcg) Cefaperazone (Cs) (75mcg), Clarithromycin (Cw)(15mcg), Sparfloxacin (Sc) (5mcg). Multidrug resistance (resistance to more ≥ 3 antibiotics tested) was noted [21]. Result was interpreted as sensitive – inhibition zone ≥ 18mm, intermediate - inhibition zone 13 - 17 mm and resistance - inhibition zone < 13 mm [22]. *Aeromonas hydrophila* (ATCC 7966) were used as controls.

4. **Multi-Antibiotics resistance index of biofilm isolates:** This was carried out as described with slight modification [23] .MARI = resistant antibiotics ÷ total antibiotics tested. MARI values > 0.2 indicate existence of isolate from high – risk contaminated source with frequency use of antibiotics while values ≤ 0.2 show bacteria from source with less antibiotics usage [24].

RESULTS AND DISCUSSION:

Isolation and identification of *A. hydrophila* in urine: In contrast to the large number of publication on the role of *Aeromonas hydrophila* causing infection in animals, humans, birds, fish, there are few papers handily the effect of *Aeromonas hydrophila* in urine. In this study urine samples were collected from private hospital in Tiruchengode for the isolation and identification of *Aeromonas hydrophila.*

According to morphological and biochemical characters, 75 isolates (50%) were identified to be *Aeromonas hydrophila* that grow on Starch ampicillin agar (SAA medium) after 24 hr incubation at 37°C. These colonies were Circular, Convex, Opaque, raised, glistering colonies with entire edge, Yellow to honey colored, amylase positive colonies (clear zone surrounding the colony) Gram negative, motile, rod shaped, facultative anaerobes and oxidase and catalase positive. White to pale pink, round and convex colonies appeared on nutrient agar (**Figure 2**), (**Table 1**).

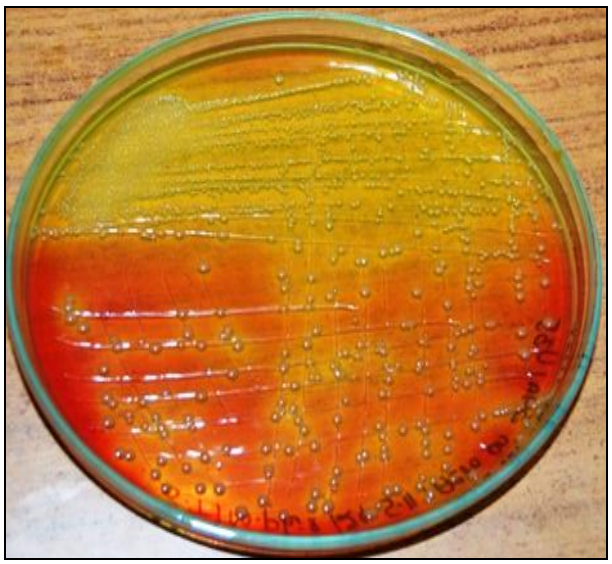

FIG. 2: A. *HYDROPHILA* ON SAA MEDIUM

TABLE 1: BIOCHEMICAL CHARACTERIZATION OF *A.HYDROPHILA* ISOLATED FROM URINE SAMPLES

S. No.	Tests	*Aeromonas hydrophila* (75 isolates)
1	Gram staining	Gram (-)
2	Motility	Motile
3	Oxidase	Purple color
4	Catalase	+
5	Indole	+
6	Methyl red	+
7	VP	+
8	Citrate	+
9	TSI	A/Ak,H_2S^+,G^+
10	Urease	+
11	Gelatin	+
12	Glucose	+,Gas^+
13	Sucrose	+
14	Lactose	-
15	Maltose	+
16	Mannitol	+
17	Mannose	+
18	Xylose	+
19	Dextrose	+
20	Nitrate	+
21	ONPG	-
22	0% NaCl	+
23	1%Nacl	+
24	6%Nacl	-

A/Ak → Acid bud and alkaline slant, H_2S→hydrogen sulfide, G^+→Gas production.

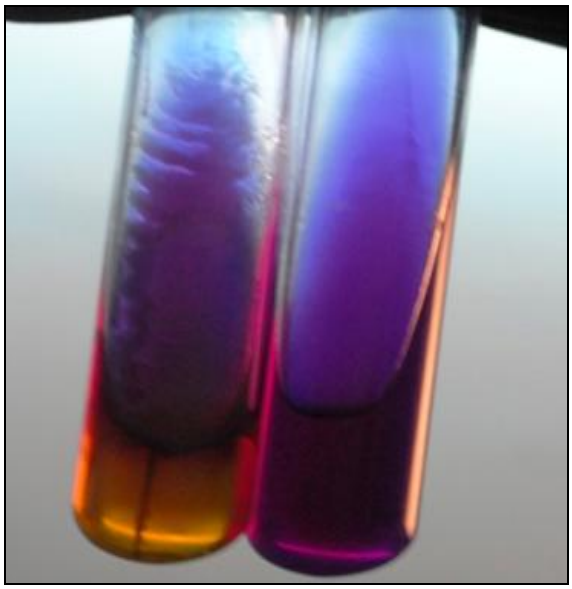

FIG. 3: *A. HYDROPHILA* ON KAPER'S MULTITEST MEDIUM

From **figure 3**, all isolates were confirmed in Kaper's multitest medium (Violet colour) was turned into acid butt (Yellow) and alkaline slant (violet) within 7 hours.

Slime production (Congo Red Agar plate Method): In this study, Congo Red Agar was used for preliminary screening of the isolates for slime production. The 50 (67%) isolates of *Aeromonas hydrophila* were positive for slime production and 25 (33%) isolates were considered as negative as a result of formation of black consistent crystalline colonies (**Figure 4)**.

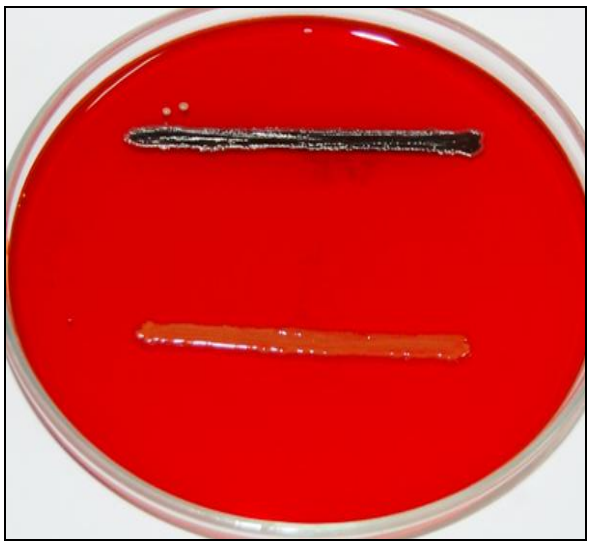

FIG. 4: *IN- VITRO* DEMONSTRATION OF SLIME PRODUCTION ON CRA MEDIUM. Slime positive- Black consistent crystalline colonies, Negative- Colorless (or) red colonies.

Gram negative opportunistic pathogen *Pseudomonas aeruginosa* can form three different types exopolysaccharides which can form the EPS matrix encasing the biofilm is another example complex nature of biofilm. Various environmental factors influence biofilm formation which includes pH, temperature, osmolarity, iron, and oxygen and growth medium composition.

Cations (sodium, calcium, lanthanum, ferric iron) influence biofilm formation. Higher amounts of biofilms were produced as the concentrations of these ions increased, presumably by reducing the repulsive forces between the negatively charged bacterial cell surface and the solid surface onto which biofilm is formed. Increase in nutrient concentration correlated with an increase in the number of attached bacterial cells forming biofilm [25].

From this result, we could conclude that the identification of *Aeromonas hydrophila* from urine was responsible for the production of slime. This slime was considered as a starting stage of biofilm and latterly leads to severe complicated disease in the human beings (**Table 2**).

Biofilm formation on the standard tube method (STM): This method is used for the quantitatively analysis of the isolates for biofilm formation (**Figure 5**).

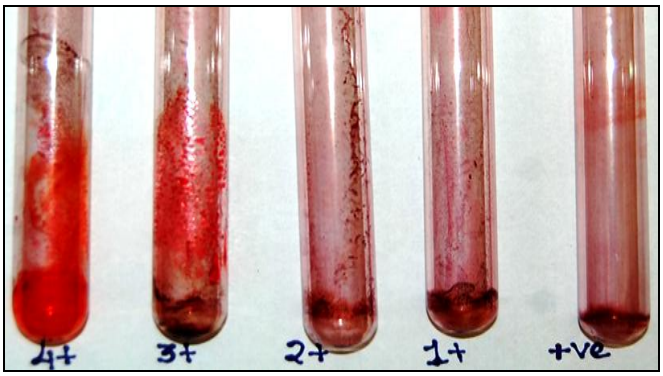

FIG. 5: FORMATION OF BIOFILM *IN VITRO* BY *A. HYDROPHILA* STAINED WITH SAFRANIN

TABLE 2: SLIME PRODUCTION

Slime production on Congo Red Agar plate method				
Isolates	**Positive**	**%**	**Negative**	**%**
Aeromonas hydrophila (75)	50	67	25	33

All Positive slime produced isolates (50) derived from above method were further studied for biofilm formation in STM method. However, STM helped in grouping all the isolates into weak, moderate and strong biofilm producers.

In this current study, 9(18%) of the isolates were weak producers (+ve, 1+) with observed on inner side of the tube. 20(40%) moderate producers (2+, 3+) and 21(42%) are strong biofilm producers (4+) (**Table 3**).

TABLE 3: BIOFILM FORMATION

Biofilm formation in standard tube method (STM)			
Isolates	**Strong producers(4+,3+)**	**Moderate (2+,1+)**	**Weak producers (+ve)**
***A.hydrophila* (75)**	**21(42%)**	**20(40%)**	**9(18%)**

This was in accordance with the investigated on biofilm formation among bacteria isolated from clinical samples in Pakistan and were able to classify the isolates into weak, moderate and strong biofilm producers. The growth of *P. aeruginosa* in biofilm enhanced its potential to form new biofilm, presumably indicating that passage in biofilm induces gene expression cascade which results in increased amount of biofilm formation[26]. EPS constitutes the primary matrix of biofilm and it may account for 50% to 90% of the total organic material of a formed biofilm. ESP may vary widely in chemical and physical property depending the microorganism(s) concerned, organic material available and subtratum involved onto which biofilm is formed. The level and type of ions bound by the EPS depends on its ionic properties, which in turn contributes to the structure and strength of the biofilm [27, 28].

Multi-Drug Resistance (MDR) patterns of biofilm isolate: In the present study, The 21 isolates of strong biofilm producers from urine samples were tested against 12 antibiotics. The results showed that the existence of multiple drug resistance among *Aeromonas hydrophila* isolates. All the 21 isolates showed highest resistance (100%) to Cephadroxil, Roxithromycin, Ampicillin /Cloxacillin, Cefuroxime, Cephotaxime, Cefaperazone, and Clarithromycin.The high resistance towards Roxithromycin (86%), Amikacin (70%), Azithromycin (70%) and low resistance to Ciprofloxacin (43%), Gentamicin (34%) (**Table 4**).

TABLE 4: PERCENTAGE OF MDR IN *AEROMONAS HYDROPHILA*

S. No	Isolates	At	Ak	G	Cf	Cq	Cu	Ro	Ax	Ce	Cs	Cw	Sc
1	U-1	S	R	S	S	R	R	R	R	R	R	R	R
2	U-2	R	R	R	R	R	R	R	R	R	R	R	R
3	U-3	S	S	S	S	R	R	R	R	R	R	R	R
4	U-4	R	R	S	S	R	R	R	R	R	R	R	S
5	U-5	S	R	S	S	R	R	R	R	R	R	R	S
6	U-6	R	R	S	S	R	R	R	R	R	R	R	R
7	U-7	R	R	R	R	R	R	R	R	R	R	R	R
8	U-8	R	R	R	R	R	R	R	R	R	R	R	S
9	U-9	R	R	S	S	R	R	R	R	R	R	R	S
10	U-10	R	S	R	R	R	R	R	R	R	R	R	R
11	U-11	R	R	S	S	R	R	R	R	R	R	R	S
12	U-12	S	S	S	S	R	R	R	R	R	R	R	S
13	U-13	R	R	S	R	R	R	R	R	R	R	R	R
14	U-14	R	S	S	R	R	R	R	R	R	R	R	R
15	U-15	R	R	R	R	R	R	R	R	R	R	R	R
16	U-16	R	R	R	R	R	R	R	R	R	R	R	R
17	U-17	R	R	R	R	R	R	R	R	R	R	R	R
18	U-18	R	S	S	S	R	R	R	R	R	R	R	S
19	U-19	S	S	S	S	R	R	S	R	R	R	R	S
20	U-20	S	S	S	S	R	R	S	R	R	R	R	S
21	U-21	S	S	S	S	R	R	S	R	R	R	R	S
	%	67	70	34	43	100	100	86	100	100	100	100	53

S-Sensitive, R-Resistant

Alarming increase in resistance of *Aeromonas* spp. to various antibiotics is of significance to public health.Various research on antibiotics resistance of *Aeromonas* spp. isolated from food, clinical samples, European rivers, treated and untreated water, fish gut and fresh water fish have been carried out. Our results revealed that previous study of *Aeromonas hydrophila* showed a resistance pattern to different antibiotics, particularly Ampicillin and Penicillin. The resistance is an indication of the presence of Beta Lactamases, common in bacterial pathogens in polluted water environment, in that the rich nutrients like calcium, magnesium and chlorides, and there is a strong correlation between the existence of these nutrients and *Aeromonas* spp. counts in brackish water

environment. The presence of these minerals stretches bacterial cell walls facilitating the transfer of genetic material, particularly the transfer of antibiotic resistance plasmids**.** In this study, the majority of biofilm forming *Aeromonas hydrophila,* showed 100% resistant to different antibiotics, particularly to the beta-lactam groups [29].

Multi-Antibiotics resistance index of biofilm isolates: All the isolates were resistant to between 4 and 10 antibiotics respectively. Three of the isolates showed resistance to 11 antibiotics while only 5 isolates showed resistant to all the tested antibiotics. The MAR index of isolates was ranging from 0.1-0.3. The MAR index value of more than 0.2 is considered to be high risk source of contamination (**Table 5**).

TABLE 5: MAR INDEX OF *AEROMONAS HYDROPHILA*

S. No.	Isolates	Resistance pattern	MAR Index
1	U-1	Ak,Cq,Cu,Ro,Ax,Ce,Cs,Cw,Sc	0.75
2	U-2	At,Ak,G,Cf,Cq,Ro,Ax,Ce,Cs,Cw,Sc,Cu	1.0
3	U-3	Cq,Cu,Ro,Ax	0.33
4	U-4	At.Ak,Cq,Cu,Ro,Ax,Ce,Cs,Cw	0.75
5	U-5	Ak,Cq,Cu,Ro,Ax,Ce,Cs,Cw	0.66
6	U-6	At,Ak,Cq,Cu,Ro,Ax,Ce,Cs,Cw,Sc	0.83
7	U-7	At,Ak,G,Cf,Cq,Ro,Ax,Ce,Cs,Cw,Sc,Cu	1.0
8	U-8	At,Ak,G,Cf,Cq,Ro,Ax,Ce,Cs,Cw,Cu	0.91
9	U-9	At,Ak,Cq,Cu,Ro,Ax,Ce,Cs,Cw	0.75
10	U-10	At,G,Cf,Cq,Cu,Ro,Ax,Ce,Cs,Cw,Sc	0.91
11	U-11	Cq,Cu,Ro,Ax,Ce,Cs,Cw,	0.75
12	U-12	At,Ak,Cf,Cq,Cu,Ro,Ax,Ce,Cs,Cw,Sc	0.58
13	U-13	At,Ak,Cf,Cq,Cu,Ro,Ax,Ce,Cs,Cw,Sc	0.91
14	U-14	At,Cf,Cq,Cu,Ro,Ax,Ce,Cs,Cw,Sc	0.83
15	U-15	At,Ak,G,Cf,Cq,Ro,Ax,Ce,Cs,Cw,Sc,Cu	1.0
16	U-16	At,Ak,Cf,Cq,Ro,Ax,Ce,Cs,Cw,Sc,Cu,G	1.0
17	U-17	At,Ak,G,Cf,Cq,Ro,Ax,Ce,Cs,Cw,Sc,Cu	1.0
18	U-18	At,Cq,Cu,Ro,Ax,Ce,Cs,Cw	0.66
19	U-19	Cq,Cu,Ax,Ce,Cs,Cw	0.5
20	U-20	Cq,Cu,Ax,Ce,Cs,Cw	0.5
21	U-21	Cq,Ro,Ax,Ce,Cs,Cw	0.5

In the present study, the percentage occurrence of antibiotic resistance of MAR index was compared. Based on the multiple drug resistant index, MARI values > 0.2 indicate existence of isolate(s) from high – risk contaminated source with frequency use of antibiotics (s) while values ≤ 0.2 show bacteria from source with less antibiotics usage. This value (MARI > 0.2) shows indiscriminate use of antibiotics among rural dwellers in Tiruchengode area as the value was > 0.2 for the isolates (**Figure 6**).

The study showed similar results with the investigated resistance of *A. hydrophila* from clinical isolates obtained from children. 95 % (n = 20) of total isolates (n = 21) have MAR index value greater than 0.2 depicting high level of antibiotic resistance due to either indiscriminate use of antibiotics or horizontal gene transfer. It could also be combination of the two factors.

The *A.hydrophila* isolated from Turkish water showed MAR index between 0.2 and 0.8 unlike *A.sobria* that have high values.

Hence, *Aeromonas* spp. most especially *A. hydrophila* have exhibited high levels of antibiotics resistance in the environment and clinical isolates.

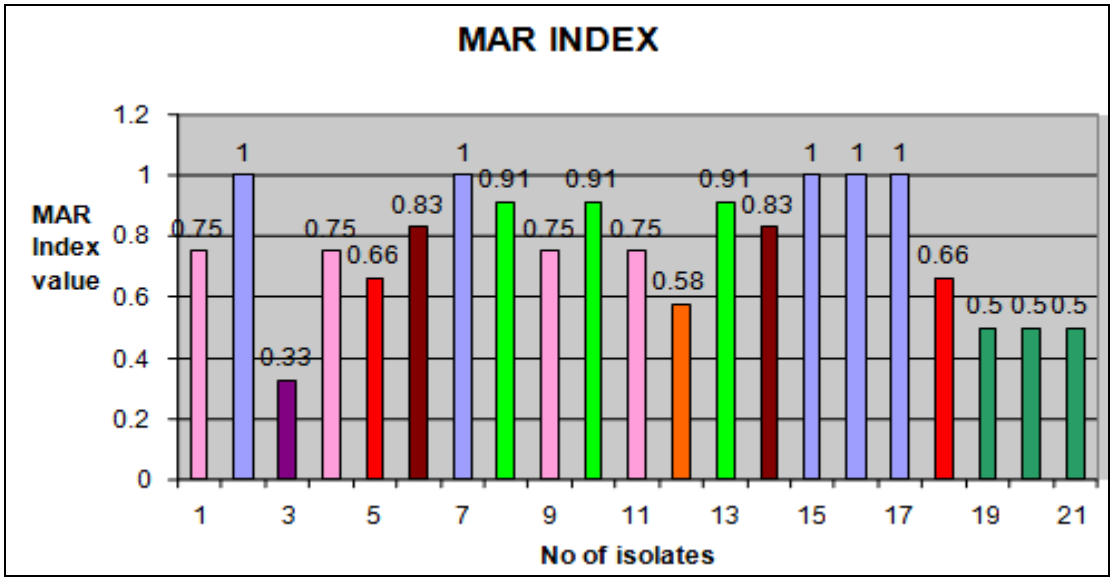

FIG. 6: MULTI-ANTIBIOTICS RESISTANCE INDEX

CONCLUSION: In this work, we concluded that the persisting nosocomial bacteria are present in liquid fluid (Urine) of human body and formed the colonization in that due to forming of independent biofilm forming pathway. Biofilms development is a multi-factorial involving polysaccharide, protein, and DNA components, which is maintained by various regulating factors.

Biofilm formation on medical devices is considered as a virulence factor and they pose a challenge in clinical settings as biofilm protect bacteria from antibiotics and host immune system. It is often impossible or undesirable to remove prosthetic device in use which may be necessary for eradication of biofilms.

There is dynamic research activity in the emerging field of biofilm as it has been identified to be of paramount importance in public health because of their critical role in many infectious diseases and in a variety of infections related to medical devices. As is the case of many areas of biological sciences, *in vivo* biofilms are much more complex and difficult to study.

However, current knowledge in bacterial biofilm provides a strong foundation to undertake a broad multidisciplinary approach that is needed to fully rationalize the clinical significance of biofilm, understand the molecular basis of the disease caused by biofilms and rational approach to eradicate biofilm. A completely novel approach to combat antibiotic resistance of bacteria in biofilm is underway.

Instead of searching for new antibiotics, the researchers have questioned whether it is possible to rejuvenate older antibiotics so that these become more effective against the resistant bacteria. In our study the urine samples were collected from commonly infected person was highly infected by biofilm forming *Aeromonas hydrophila*. The infected samples were containing higher percentage of biofilm formation and this virulence character finally, leads to the multi-drug resistant.

In biofilm condition, the microbial population exhibits the multi-drug resistance towards to different antibiotics. So that, in this current process the importance is given to determining the multi-drug resistance patterns of biofilm (virulence character) forming *Aeromonas hydrophila* from urine samples. In future aspects, we should detaily study about the relationship between the nature of drug and structure of the biofilm by using novel techniques etc.

ACKNOWLEDGEMENT: The authors are highly grateful towards their respective institutes/departments for the encouragement provided to carry on research works. The corresponding author highly acknowledges Dr. P. Vijaya Lakshmi and B.T. Suresh Kumar for their constant support and guidance.

REFERENCES:

1 Altwegg M and Geiss HK: *Aeromonas* as a human pathogen. Crit.Rev.Microbiol.1989; 16: 253-286.

2 Marti Nez-Murcia A J, Saavedra M J, Mota V R, Maier T, Stackebrandt E, Cousin E: *Aeromonas aquariorum* sp. nov.,

isolated fromaquaria of ornamental fish. In International Journal of Systematic and Evolutionary Microbiology 2008; 58: 1169 -1175.

3 Alperi A, Martínez-Murcia AJ, Wenchien KO, Arturo M, Maria JS, Maria JF: *Aeromonas taiwanensis* sp. nov. And *Aeromonas sanarellii* sp. nov., clinical species from Taiwan. In International Journal of Systematic and Evolutional Microbiology 2010; 60: 2048-2055.

4 Castro Escarpulli G,Figueras M J, Aguilera Arreola M G, Soler L, Fern Indez-Rendon E, Aparicio GO, Guarro J, Chac NM: Characterization of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico. In International Journal of Food Microbiology 2003; 84: 41-49.

5 Subashkumar R, Thayumanavan T, Vivekanandhan G, Lakshmana perumalsamy P: Occurrence of *Aeromonas hydrophila* in acute gastroenteritis among children. In Indian Journal Med Res. 2006; 123: 61– 66.

6 Rodney M D: Biofilm Formation: A clinically relevant microbiological process. In Clinical Infectious Diseases 2001; 33: 387 -392.

7 Merino S, Rubires X, Knochel S, and Tomas JM: Emerging pathogens *Aeromonas* spp. Int.J.Food.Microbiol.28: 157-168.

8 Stoodley P, Sauer K, Davies DG, and costerton JW: Biofilm as complex differentiated communities. Annu.Rev.Microbiol.56; 187-209.

9 Gottenbos B, Vandermei HC, and Busscher HJ: Models for studying initial adhesion and surface growth in biofilm formation on surfaces. Methoda Enzymol.1999; 310: 523-534.

10 Garrett TR, Bhakoo M,and Zhang Z:Characterisation of bacterial adhesion and removal in a flow chamber by micromanipulation measurements.Biotechnol.Lett.2008; 30(3): 427-433.

11 Costerton JW, Stewart PS, Greenberg EP: Bacterial biofilm: common cause of persistent infection. Science 1999; 284:1388-1392.

12 Antunes L, Caetano M, Ferreira RBR: Biofilms and bacterial virulence. Rev Med Microbiol 2011; 22:12-16.

13 Rasmussen TB, Givskov M: Quorum sensing drugs as anti-pathogenic drugs. International J Med Microbiol 2006; 296:149-161.

14 Mah TC, O Toole G: Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001; 9:34-39.

15 Palumbo AS, Marino CW, Williams AC, Buchanan RL, and Thraoer DW: Starch Ampicillin Agar for the quantitative detection of *Aeromonas hydrophila*. Appl. Environ. Microbial.1985; 50: 1027-1030.

16 Kaper JB, Lockman H, Colwell RR: *Aeromonas hydrophila*: Ecology and toxigenicity of isolates from an estury.J.Appl.Bacteriol.50: 359-377.

17 Thenmozhi S, Rajeswari P, Kalpana M, Hemalatha M, Vijayalakshmi P: Antibactericital activity of Silver nitrate on Biofilm forming *Aeromonas* spp. isolated from Drinking Water.International journal of pure and applied Bioscience 2013;1(6):117-125.

18 Christensen F R, *et al*, Biofilm structure - an important and neglected parameter in wastewater treatment. In Water Science Technology, 1989; 21: 805.

19 Yazdani R, Oshaghi M, Havayi A, Pishva E, Salehi R, Sadeghizadeh M, Foroohesh H: Detection of *Ica AD* gene and biofilm formation in *S. aureus* isolates from wound infections Iranian. In Journal of Public Health, 2006; 35: 25 - 28.

20 Bauer A, Kirby W, Sherris j, *et al*.,: Antibiotic susceptibility testing by a standardized single disk method. In America Journal of Clinical Pathol.1966; 36: 493 - 496.

21 Oteo J, Lázaro E, Abajo FJ, Baquero F, Campos J: Antimicrobial-resistant invasive *Escherichia coli*, Spain. In Emerg Infect. Dis, 2005; 11(4): 546 - 553.

22 Okonko IO, Donbraye LO, B. Ijandipe LA, Ogun AA, Adedeji AO, Udeze AO: Antibiotics sensitivity and resistance patterns of uropathogens to nitrofurantoin and nalidixic acid in pregnant women with urinary tract infections in Ibadan, Nigeria. In Middle - East Journal of Sci. Research.2009; 4(2): 105-109.

23 Matyar F, Kaya A, Dincer S: Distribution and antibacterial drug resistance of *Aeromonas spp.* from fresh and brackish waters in Southern Turkey. Annals of Microbiology. 2007; 57: 443 - 447.

24 Krumperman PH: Multiple antibiotic indexing of *E.coli* to identify high-risk sources of fecal contamination of foods. In Applied Environmental Microbiology. 1985; 46: 165 -170.

25 Ghafoor A, Hay ID, Rehm BHA: Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. Appl Environ Microbiol. 2011; 77: 5238–5246.

26 Hassani A, Javaid U, Kaleemi F, Omairiii M, Khalidi A, Muhammad I: Evaluation of different detection methods of biofilm formation in the clinical isolates. In Brazilian Journal of Infectious Diseases, 2011; 15(4): 345 - 350.

27 Hossain A: Growth in biofilm enhances potential to form new biofilm by *Pseudomonas aeruginosa*. Life Sci J.2013; 10:356-359.

28 Kolter R, Biofilms in lab and nature: a molecular geneticist's voyage to microbial ecology. International Microbiol 2010; 13:1-7.

29 Mansour FA, Zaky MM, Salem MA: Environmental and health significance of toxigenic *Aeromonas sobria* and *A.hydrophila* in polluted water.26th Arican health sciences congress. 2005.

How to cite this article:
Thenmozhi S, Rajeswari P, Suresh Kumar BT, Saipriyanga V and Kalpana M: Multi-drug resistant patterns of biofilm forming *aeromonas hydrophila* from urine samples. *Int J Pharm Sci Res* 2014; 5(7): 2908-18.doi: 10.13040/IJPSR.0975-8232.5 (7).2908-18.