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Research Article

Biofilm formation and effect of disinfectant on the isolates obtained from aquatic ecosystem

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Abstract:
Aeromonas spp. are common aquatic micro-organisms that occur in seawater, irrigation water, river water, brackish water, fresh water, ground water, spring water, industrial and domestic waste water. *Aeromonas* spp. is associated gastrointestinal diseases, mainly diarrhea, septicemia, wound infections and diseases of amphibians, reptiles, frog, fish etc. Every month water samples were collected from well (village pal), river (Tapti river), tap (Surat Municipal Cooperation), mineral (Aquafina), sea (brackish) and swimming pool (chlorinated) of Surat city during year 2009 to 2010 and analyzed microbiologically. Isolates like *Aeromonas hydrophila, Aeromonas salmonicida, Aeromonas salmonicida sub spp. smithia, Aeromonas salmonicida sub spp. masoucida, Aeromonas schubertii, Aeromonas veronii, Aeromonas media and Aeromonas caviae* were obtained from the water samples and were further tested for their Biofilm formation and effect of disinfectant was checked on the produced Biofilm. As *Aeromonas* species are one of the Biofilm producing organism, the Biofilm production test was carried out by using polycarbonated plastic/PVC and Glass coupons deeped in three different containers like copper, steel and clay. Weight of the polycarbonated plastic coupons and glass coupons were noted and at the interval of 15 days and 30 days, increase in weight of the plates and coupons showed Biofilm formation. In water distribution system these organisms produce the Biofilm and so to remove it disinfectant treatment is used. Disinfectant treatment using (chlorine tablets) containing sodium dichloro isocyanurate, having 10 ppm of chlorine commercially available to disinfect drinking water. Serial plate count was done using M-Aeromonas selective media to get specific number of *Aeromonas* species. The hanging glass coupons were removed at time interval of 30min, 60min, 90min (with treatment of disinfection) and 0 min (without disinfection). The coupons were stain by using fluorescent dye Acridine orange to get number of viable and non viable cells forming Biofilm. We can also conclude that the Biofilm produced by them in a water distribution system i.e. pipe lines or domestic vessels can be controlled easily by disinfectants within an hour.

Keywords: Aquatic ecosystem, drinking water, Biofilm, Disinfectant.

Introduction:

Aeromonas spp. are common aquatic micro-organisms that occur in seawater, irrigation water, river water, brackish water, fresh water, ground water, spring water, industrial and domestic waste water. [6] The wide distribution of Aeromonas species in different aquatic ecosystems underlines their capacity to adopt with environments in different tropics levels. Several studies have shown that the phenospecies But few studies show that the count of Aeromonas species are usually found more during summer than in winter [10]

There are eleven named speices and their subspecies commonly found in aquatic environment which are associated with diarrheal illness and can cause infections and septicemia. Aeromonas species are gram negative, motile, facultative anaerobic, rod shaped, Oxidase positive bacteria of the recently assigned family Aeromonadaceae [1, 4].

In aquatic ecosystem groups of microorganisms cooperatively from Biofilm on the various support systems may be pipe line, fishes, aquatic plant etc. Microorganisms remain in the aquatic system either as a suspended form or as a Biofilm [8]. A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface.

Material and Methods:

All the requisite materials used for the sample collection were previously sterilized and of standard quality viz. Bottles use to collect water sample. All media use for isolation and identification of Aeromonas spp. was use of standard quality viz. Hi media, Mumbai. For the biofilm formation test the standard coupons were used and for Disinfectant treatment (chlorine tablets) containing sodium dichloro isocyanurate, having 10 ppm of chlorine commercially available to disinfect drinking water was used.

The study was carried out during September -2009 to September -2010.

(A) List of samples to be collected:

1. Well water (Village: Pal)

2. River Water (Tapti river, untreated)

3. Tap Water (SMC)

4. Mineral Water. (Aquafina)

5. Sea Water. (Brackish)

6. Swimming-Pool Water. (Adajan-surat)

(B) Frequency of Samples to be Collected:

Every month six samples were collected. As Aeromonas are found in drinking water; the samples need to be collected from the similar sites every month to check seasonal variations.

(C) Volume:

Two liters water sample was collected in sterile plastic bottles from different site as mention above.

(D) Handling and transport:

After collection of water sample bottles were transported to the institutional laboratories for further processing immediately.

[A] Isolation and characterization:

All samples were analyzed microbiologically and physicochemically using standard methods in term of their quality and quantity [3, 8]. Quantitative analysis was done by Membrane filtration procedure was used for the enumeration of Aeromonas species by using M-Aeromonas selective medium containing Ampicillin (Hi-Media, Mumbai). Qualitative analysis was done by observing its colony characteristics and growth characteristics was studied using Nutrient Agar plate, Mac Conkey's Agar and on Aeromonas selective media Rippey Cabelli's agar (Hi Media, Mumbai) [5].The isolates were identified from their cultural and biochemical characteristics [1, 2]. Final confirmatory test was done using Sheep Blood Agar (Hi Media, Mumbai) to check the pathogenesis of the obtained isolates [6]. All of them were then incubated at 250C - 370C for 24 hours.

[B] Biochemical profiling:

The biochemical profiling was carried out by performing various biochemical tests viz, Carbohydrate utilization test, Oxidative fermentation

test, Citrate utilization test, Gelatin liquefaction test, Indole production test etc. The isolates were also characterized using multi test media such as T. S. I. agar Slant, MR-VP medium and motility lysine agar medium. All the media were inoculated with the loop full of culture by aseptic transfer technique or stabbing technique. The inoculated test media were incubated at 370C for 24-48 hours.

[C] Identification:

The isolates were then identified from their morphological, cultural and biochemical characteristics using standard references [1, 4, 11].

[D] Biofilm formation:

As Aeromonas species are one of the Biofilm producing organisms, the Biofilm production test was carried out by using polyvinyl chloride (PVC) and Glass coupons deeped in three different containers like copper, steel and clay. Weight of the polyvinyl chloride coupons and glass coupons were noted and at the interval of 15 days and 30 days, formation of the film was checked by observing the increase in weight of the plates and coupons. From the deeped coupons, the film was scraped and collected in a

sterile test tube and distilled water was added and vortex. The enumeration of the Aeromonas species was done using M-Aeromonas selective media [8].

[E] Disinfectant treatment:

In water distribution system these organisms produce the Biofilms and so to remove it disinfectant treatment was used. Disinfectant treatment using (chlorine tablets) containing sodium dichloro isocyanurate, having 10 ppm of chlorine commercially available to disinfect drinking water. Serial plate count was done using M-Aeromonas selective media to get specific number of Aeromonas species. The hanging glass coupons were removed at time interval of 30min, 60min, 90min and was stain by using fluorescent dye Acridine orange [9]. The slides were observed under fluorescent microscope, at 600 x magnification (H110 FLUORESTOR microscope equipped with 2071 H vertical fluorescence illuminator) to check the viable and non viable bacterial cell along with control [7, 9].

Result and discussion:

There are 22 different species of Aeromonas known till date, out of which 11 species are commonly found in aquatic environment. According to the research work, 8 different isolates of Aeromonas spp. were isolated from the water samples under study viz. A. hydrophila A. salmonicida subspp. salmonicida, A. salmonicida subspp. smithia, A. salmonicida subspp. masoucida, A. veronii, A. schubertii, A. media, A. caviae.

Biofilm formation:

The coupons made of PVC and glass was dipped in different container like mud, steel and copper were used for Biofilm study.

Table: 1 - Change in weight of coupons.

PVC sheet:

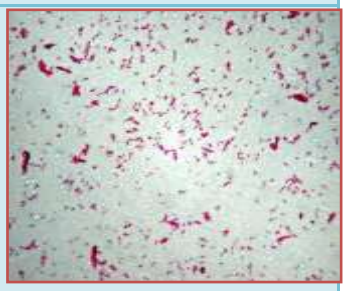
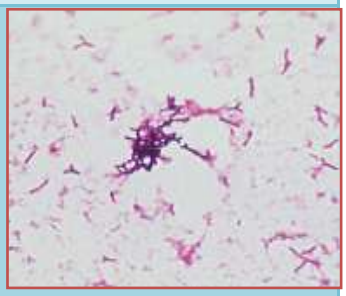
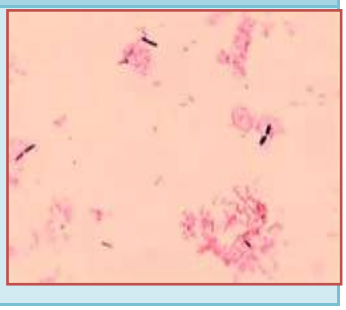
Container	Before	After 15 days	After 30 days	Difference
Mud	16.351 gm	16.402 gm	16.457 gm	0.055
Steel	15.981 gm	16.056 gm	16.134 gm	0.078
Copper	15.941 gm	16.031 gm	16.122 gm	0.091

Glass slide:

Container	Before	After 15 days	After 30 days	Difference
Mud	5.677 gm	5.682 gm	5.688 gm	0.006
Steel	5.982 gm	5.998 gm	6.061 gm	0.063
Copper	5.457 gm	5.459 gm	5.499 gm	0.040

Increasing weight of coupons shows the Biofilm formation.

Table: 2 - Heterotrophic count from Biofilm using Nutrient agar.

Sample	Poly carbonated plates		Glass coupons		Figure
	Average CFU / ml.	Morphological diversity in Biofilm	Average CFU / ml.	Morphological diversity in Biofilm	
Mud vessel	1135 x 10^6	Gram positive and Gram negative, rod shape bacteria appear singly and in pairs	188 x 10^6	Gram positive and Gram negative, rod shape bacteria appear singly and in pairs	
Steel vessel	42 x 10^6	Gram positive and Gram negative, rod shape bacteria appear singly and in pairs	165 x 10^6	Gram positive and Gram negative, rod shape bacteria appear singly and in pairs	
copper vessel	28 x 10^6	Gram positive and Gram negative, rod shape bacteria appear singly and in pairs	123 x 10^6	Gram positive and Gram negative, rod shape bacteria appear singly and in pairs	

The coupons made of PVC and glass was dipped in different container like mud, steel and copper were used for Biofilm study. The biofilm did form on the coupons which was note down by the increase weight of coupons. Another finding was that the formation of biofilm was less in copper vessel than compared to steel amd mud. So use of copper vessels for storage of water should increase, which can be a good innovative and cheap house hold method.

Disinfectant treatment:

Disinfectant treatment using (chlorine tablets) containing sodium dichloro isocyanurate, having 10 ppm of chlorine commercially available to disinfect drinking water. Serial plate count was done using M-Aeromonas selective media to get specific number of *Aeromonas* species. The hanging glass coupons were removed at time interval of 30min, 60min, 90min (with treatment of disinfection) and 0 min (without disinfection). The coupons were stain by using fluorescent dye Acridine orange to get number of viable and non viable cells forming Biofilm.

Table: 3 - Number of Aeromonas from Biofilm using M-Aeromonas selective media.

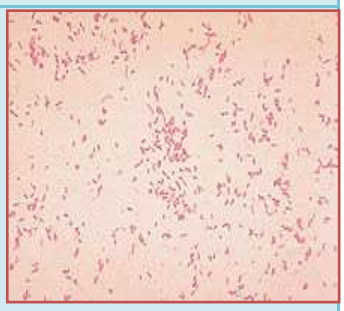
Sample	Poly carbonated plates		Glass coupons		Figure
	Average CFU / ml.	Morphological diversity in Biofilm	Average CFU / ml.	Morphological diversity in Biofilm	
Mud vessel	965 x 10^6	Gram negative, rod shape bacteria with rounded end.	128 x 10^6	Gram negative, rod shape bacteria with rounded end.	
Steel vessel	37 x 10^6	Gram negative, rod shape bacteria with rounded end.	37 x 10^6	Gram negative, rod shape bacteria with rounded end.	
copper vessel	20 x 10^6	Gram negative, rod shape bacteria with rounded end.	24 x 10^6	Gram negative, rod shape bacteria with rounded end.	

Fig: 1 – Fluorescent staining using by Acridine orange

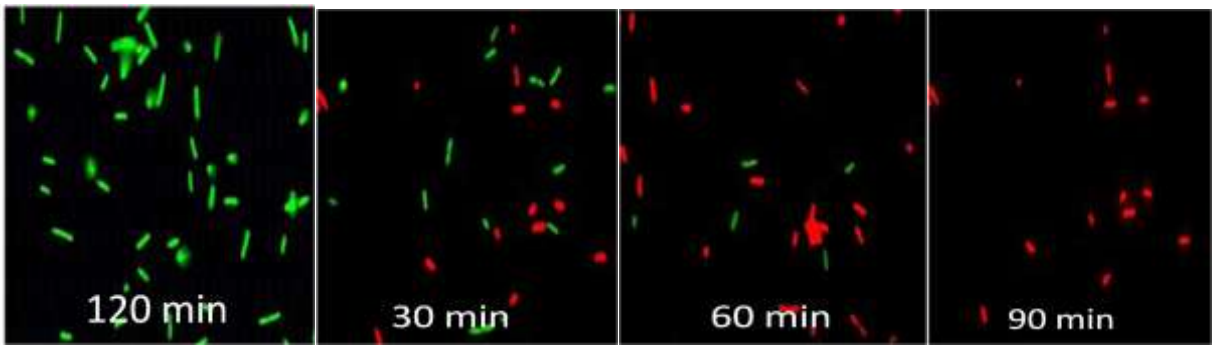
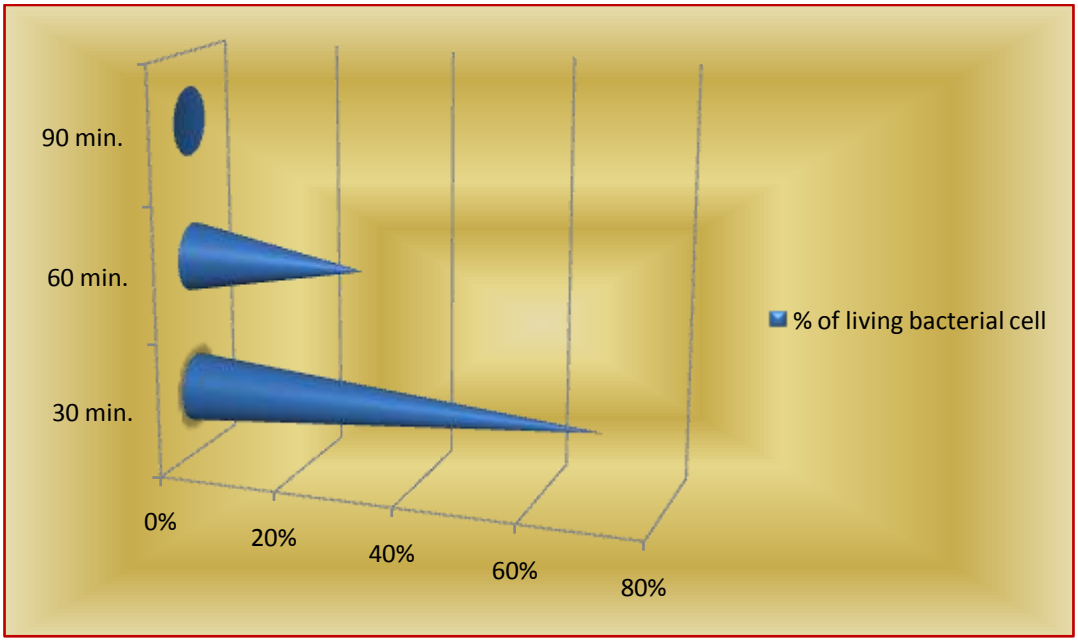


Fig: 2 - Percentage of living bacterial cell by disinfectant treatment.



From the graphical presentation of time kill assay we can conclude that during the treatment of disinfectant, in 30 min mostly the cells are in living state, in 60 min the number of dead cells increases while in 90 min mostly all cells are dead due to the treatment of disinfectant. In 0 min, without any treatment all cells are living and by performing gram staining, it shows presence of gram negative short rods with rounded ends, which appear singly

Conclusion:

From the above study total 166 isolates were obtained which contain eight different species of *Aeromonas.* Many different species of *Aeromonas* were isolated namely *Aeromonas hydrophila, Aeromonas Salmonicida, Aeromonas Salmonicida subsp. smithia, Aeromonas Salmonicida subsp. masoucida, Aeromonas schubertii, Aeromonas veronii, Aeromonas media, Aeromonas caviae* were found from the different water samples. The highest number of isolates obtained was of *Aeromonas hydrophila, Aeromonas salmonicida subsp. smithia and Aeromonas salmonicida.* Indirectly fish is a diet food of human, so infection can spread through fish to humans. The seasonal variation was observed in the obtained isolates, the number of *Aeromonas* species were more during warmer months then in winter. April May and June gave the highest number of isolates.

The study proves that the obtained isolates of *Aeromonas* species were able to form Biofilm which indicates that they are generally more resistant to disinfection and different antibiotics. The disinfection treatment using (chlorine tablets) containing sodium dichloro isocyanurate available in market to disinfect drinking water, showed that mostly the speices of *Aeromonas* are able to resist the chlorination treatment for 1 hour. Thus a mear suggestion is that the presence of *Aeromonas species* in drinking water needs public health appraisal and further work should be undertaken to permit revaluation of standards for the quality of drinking water. Even some solution should be made to solve the problem of biofilm formation in different water distribution systems. Thus it serves to check the quality of water and for the purpose of public health.

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