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Research Article

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Antibactericital activity of Silver nitrate on Biofilm forming *Aeromonas spp.* Isolated from Drinking water

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ABSTRACT

The occurrence of biofilm forming Aeromonas spp. in drinking water in the Tiruchengode area was monitored. Aeromonads cause traveler's diarrhea in millions of peoples; particularly traveler's visiting less developed regions in Asia. The main source of infection is contaminated water.A total of 100 water samples were collected from several sampling sites during a period of one year. Among these only 50 isolates were found to be positive for Aeromonas spp. The Starch-Ampicillin agar were used as a selective presumptive isolation medium for the isolation of bacterial isolates from the samples and confirmed as Aeromonas hydrophila 30(60%), Aeromonas caviae 20(40%) were determined by using standard biochemical analysis according to Bergey's manual of systematic Bacteriology. Susceptibility tests were carried out according to the criteria of national committee for clinical laboratory standards. MIC's were obtained by agar diffusion method. Slime/Biofilm producing isolates were studied on Congo Red Agar (CRA) method. The detection of the presences of such virulence factors is a better indicator of the potential health rish, Infection due to bacterial pathogen with such virulent factors through contact with water (or) infected person act as a health hazards to humans. So that, In our study slime/biofilm forming isolates were detected and inactivated by using Silver nitrate. The samples taken from the drinking water in Tiruchengode area were found to contain very diverse biofilm forming Aeromonas populations that can pose a risk for the residents of the city.

Key Words: *Aeromonas spp., Antibiotic sensitivity test, Biofilm, Antibactericital activity of Silver nitrate.*

INTRODUCTION

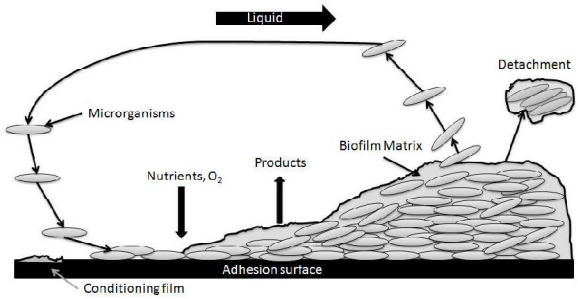
The importance of detection of *Aeromonas* spp. has increased in recent years due to their emergent human pathogenic properties[1]. *Aeromonas hydrophila- Hydro*-water: *philos*-loving, *hydrophila* →Water loving. Althought *Aeromonas* was discovered more than a century ago, its role in a variety of human diseases was proved only during the past three decades.The members of the genus *Aeromonas* are Gram-negative, oxidase-positive, facultative anaerobic, glucose fermenting, straight rods with rounded ends of bacteria. But sometimes can appear as coccobacilli or with rounded ends, Cells are 0.3-1.0 x 1.0-3.5 µm and can occur singly, in pairs, or even as short chains[2].

Mesophilic *Aeromonas* spp. are common organisms in the environment, especially in water and sewage and also occur in untreated and treated Drinking water. Neither the sources nor the routes of infection are known though the organisms are most often isolated from man in summer when the bacillary population becomes the highest in aquatic habitats[3]. *Aeromonas* spp. can have different adhesive abilities, depending on environmental conditions. A single polar flagellum facilitates both adhesion and invasion of human and fish cell lines[4]. In viscous environments or over surfaces, *Aeromonads* are able to produce many peritrichous lateral flagella, which increase bacterial adhesion and are required for biofilm formation[5].

Other studies suggest that non-pillar polysaccharide adhesins also play an important role in adhesion of *Aeromonas* spp.[6].

The World Health Organization lists *Aeromonas hydrophila*, a member of the genus *Aeromonas*, as a potential waterborne pathogen. In humans, *Aeromonas* causes diarrhea, gastroenteritis and extraenteritic condition such as septicemia, wound infection, endocarditis, meningitis and pneumonia, extraintestinal infections such as peritonitis or otitis or of sites such as the eye or urinary tract. The presences of these organisms in drinking water supplies, including in those chlorinated is a reason to public health concern due to their capacity to produce toxins and colonize the biofilms[7, 8]. The number of cases of *Aeromonas* associated gastroenteritis increases during the summer and correlates with an increased number of *Aeromonads* in water systems[9, 10]. Antibiotic resistance is particularly relavant in pathogen of *Aeromonas* species in which, besides the classical resistant to β-Lactamic antibiotics,multiple resistance has been frequently identified. These bacteria can receive and transfer antibiotic resistance genes to other Gram negative bacteria. In the last few years, the biofilm structure has been revealed as an important bacterial association with a significant role in exacerbating human infection, because it provides bacteria some properties that make antibiotics treatment difficult[11].

Bacterial cells in a biofilm are surrounded by a self-synthesized, three-dimensional matrix that holds the cells together in a mass and firmly attaches the bacterial mass to the underlying surface.This matrix, referred to as the slime layer, glycocalyx, or extracellular polymeric substance (EPS) matrix, can comprise up to 90% of the biofilm biomass. In addition to its structural role, the EPS matrix provides biofilm cells with a protected microenvironment containing dissolved nutrients and secreted enzymes, as well as other biological molecules and a biotic substances originating from outside the biofilm. The EPS matrix may also contribute to the increased resistance to antibiotics and host defenses exhibited by biofilm cells. Polysaccharide is a major component of the EPS matrix in most bacterial biofilms.

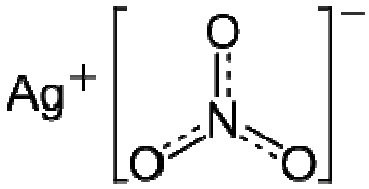


Biofilm Related Infectious Diseases

Biofilms have been found to be related to a large variety of microbial infections in the body such as urinary tract infections, middle-ear infections, formation of dental plaque, gingivitis, coating contact lenses [12] etc. Most biofilms are comprised of a variety of organisms, i.e. polymicrobial. Biofilms are best known for their role in foreign device-related infections including infections of catheters and other biomaterials used in medicine[13]. Again, the increased resistance to antimicrobial agents and infections by biofilms make the research on biofilms extremely important for medical and clinical use[14].

Silver has been known to possess bactericidal properties and silver compounds have been used for their medicinal properties for centuries as well. Several products incorporating silver have been used as topical antibacterial agents, such as silver nitrate and silver sulphadiazine Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds by manipulating their size to alter the effect. Silver has long been known for its antimicrobial properties, but its medical applications declined with the development of antibiotics. Different studies have established the bactericidal effect of nano silver against Gram negative and Gram positive bacteria, and suggested that silver nanoparticles attach to the surface of the cell membrane and disturb its function, penetrate bacteria, and release silver ions[15].

Silver nitrate



The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfydryl, ™SH) groups although other target sites remain a possibility demonstrated that amino acids such as cysteine and other compounds such as sodium thioglycolate containing thiol groups neutralized the activity of silver nitrate by contrast, amino acids containing disulfide (SS) bonds, non-sulfur containing amino acids, and sulfur-containing compounds such as cystathione, cysteic acid, L-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag1 activity. These and other findings imply that interaction of Ag1 with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved. Hydrogen bonding, the effects of hydrogen bond-breaking agents, and the specificity of Ag1 for thiol groups were discussed in greater detail.Virucidal properties might also be explained by binding to ™SH groups proposed that silver salts and other heavy metals such as copper act by binding to key functional groups of fungal enzymes. Ag1 causes the release of K1 ions from microorganisms; the microbial plasma or cytoplasmic membrane, with which is associated many important enzymes, is an important target site for Ag1 activity. In addition to its effects on enzymes, Ag1 produces other changes in microorganisms. Silver nitrate causes marked inhibition of growth of microbes and is deposited in the vacuole and cell wall as granules. Ag1 inhibits cell division and damages the cell envelope and contents of Bacterial cells increase in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibit structural abnormalities, although without any blebs (protuberances). Finally, the Ag1 ion interacts with nucleic acids it interacts preferentially with the bases in DNA rather than with the phosphate groups.[16] The objective of this study was to determine if silver nitrate inhibits the formation of biofilm producing *Aeromonas* spp. on water distribution surfaces to evaluate the potential of silver as a secondary residual disinfectant in water distribution systems.

MATERIALS AND METHODS

Study area

The objective of this study was to investigate the occurrence and inactivation of slime/biofilm(virulence factor) producing *Aeromonas* spp. in chlorinated drinking water of kaavery river in Tiruchengode area were monitored. Chlorinated kaavery river drinking water supply has been done by through pipe lines that are maded by cement materials. Prolonged storage of water in cement pipe lines is a one of the choice for the formatiom of biofilm on the innerwall of the pipe lines. So that in this study the biofilm producing isolates were identified and inactivated by using nanoparticles such as Silver nitrate were performed. It is important to highlights that the presence of virulence factor producing *Aeromonas* spp. in water system.

Collection of water samples

A total of 100 chlorinated river water samples were collected from Tiruchengode area during the year of Septemper 2011 to September 2012. The samples were collected according to the APHA Standard Methods in sterile 500ml of disposable bottles. In order to inactivate chlorine, sterile sodium thiosulphate solution was added (13.2mg/l). The samples were immediately stored under ice-cold conditions and microbiological analyses were performed within 3 h of collection[17].

Isolation and Preservation

10ml of water were inoculated in 90 ml of peptone water with 1% Nacl(w/v) at pH 8.6 adjusted with sodium hydroxide. After incubation at 37°C for 24-48 h the cultures were streaked on 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.[18] 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 and oxidase test.

Furtherly it was tested in Kaper's multitest medium.[19] Then the identified colonies were subsequently maintained in Brain Heart Infusion Agar slants (BHIA) at 4 ºC.

Morphological and Biochemical Tests

The experimental isolates of *Aeromonas hydrophila, Aeromonas caviae* was isolated from above method were subjected to their morphological studies and biochemical tests as recommended by using standard biochemical tests.

Antimicrobial Susceptibility Tests

The resistances of all strains to different antimicrobial agents were determined by the disk diffusion method as described by (NCCLS) [20]. The antibiotic and concentration ranges tested were as follows: Cephadroxil (30mcg), Cefuroxime (30mcg), Ciprofloxacin (5 mcg), Gentamicin (10 mcg), Azithromycin (15 mcg), Penicillin (10 µg), Ampicillin (10 µg), Amikacin (30 mcg), Muller Hinton agar plates were prepared and sterilized at 121ºC for 15 minutes and swabed the cultures on the plates with sterile swab. Plates were left at room temperature to remove excess of moisture. With sterile forceps, different antibiotics were placed and kept at refrigerator for 30 minutes for pre- diffusion of the disc. Then the plates were incubated at 37 ºC for 24 hours. Following incubation, the zone of inhibition in millimeter was noted, the sensitivity and resistance breakpoints were defined by the National Committee for Clinical Laboratory Standards (NCCLS) for Gram negative bacteria. *Aeromonas hydrophila* (ATCC7966), *Aeromonas caviae* (ATCC 15468) were used as controls.

Silver solution preparation

A solution of silver was prepared by adding silver nitrate to sterile dechlorinated tap water to a stock concentration of 100 mg/L (ppm) of silver ions immediately prior to the experiments. A 100 µl volume of this stock solution was added to 100 ml of the test bacterial suspension to obtain a final concentration of 100 µg/L (ppb) of silver.

Slime/Biofilm 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 [21]. The isolates were streaked to a length of 1.5 cm 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.

Antibactericital activity of silver nitrate on Slime/Biofilm forming Isolates

The antibactericital activity of silver nitrate was determined on agar plates supplemented with (0.086mg/l) of congo red dye.0.2 ml of already prepared silver nitrate solution was added in the 10 ml of BHIB (Brain Heart Infusion Broth).Then inoculated the slime producing isolates and incubated at 37˚C for 24hrs. After incubation period, the inoculated broth cultures were streaked to a length of 1.5 cm on Congo red agar medium.Then incubated at 37˚C for 24 hrs. After incubation period observed the results[22].

RESULTS AND DISCUSSION

Morphology and biochemical characteristics of bacterial isolates

In this study, we isolated a total number of 50 possible *Aeromonas* colonies from drinking water samples on the basis of morphological characters on the Starch Ampicillin Agar (SAA agar). These isolates were characterized with physiological and conventional biochemical tests. According to the morphological and biochemical characters, 30 isolates (60%) were identified to be *Aeromonas hydrophila* and 20 (40%) isolates were identified as *Aeromonas caviae* that grow on Starch ampicillin agar 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 colonies were obtained (Table 1).

Table: 1Biochemical characterization of *Aeromonas* spp. isolated from drinking water

S.No	Tests	*Aeromonas hydrophila* (30 isolates)	*Aeromonas caviae* (20 isolates)
1	Gram staining	Gram(-)	Gram(-)
2	Motility	Motile	Non-motile
3	Oxidase	Purple colour	Purple colour
4	Catalase	+	+
5	Indole	+	+
6	Methyl red	+	+
7	VP	+	-
8	Citrate	+	+
9	TSI	A/Ak,H_2S^+,G^+	A/AK,H_2S^-,G^+
10	Urease	+	+
11	Gelatin	+	+
12	Glucose	+,Gas^+	+,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

Antibiotic susceptibility

Susceptibility Patterns of *A.hydrophila* and *A.caviae* to antimicrobial agents have varied, but isolates were usually susceptible to Cephadroxil, Cefuroxime, Gentamicin. In vitro susceptibility of the *Aeromonas* isolates to a variety of antibiotics shown in (Table 2). These data revealed to 100% of the *A.hydrophila* and *A.caviae* isolates sensitive to Cephadroxil, Cefuroxime, Gentamicin. Among the 30 isolates of *A.hydrophila* 29 (97%) showed sensitive to Ciprofloxacin, 25 isolates (83%) for Azithromycin, and 29 (97%) isolates for Amikacin. Among the 20 isolates of *A.caviae* 18(90%) showed sensitive to Ciprofloxacin, 16(80%) to Azithromycin and 14 (70%) to Amikacin, while all isolates of *A.hydrophila* and *A.caviae* were resistant to Penicillin and Ampicillin property due to beta-lactamase production. These results agree with obtained by A.M. Abdel-Gwad who showed that most of the *Aeromonas hydrophila* isolates sensitive to Gentamicin, Nalidixic acid and all resistant to Penicillin, Ampicillin[23].

Table: 2 Antibiotic sensitivity test for *Aeromonas spp.*

Antibacterial agent	*Aeromonas hydrophila*				*Aeromonas caviae*			
	Sensitive		Resistant		Sensitive		Resistant	
	No	%	No	%	No	%	No	%
Cephadroxil (30mcg)	30	100	0	0	20	100	0	0
Cefuroxime (30mcg)	30	100	0	0	20	100	0	0
Ciprofloxacin (5mcg)	29	97	1	3	18	90	2	10
Gentamicin (10mcg)	30	100	0	0	20	100	0	0
Azithromycin 15mcg)	25	83	5	17	16	80	4	20
Penicillin (10µg)	0	0	30	100	0	0	20	100
Ampicillin (10µg)	0	0	30	100	0	0	20	100
Amikacin (30mcg)	29	97	1	3	14	70	6	30

Slime/Biofilm production

In this study Congo Red Agar was used for preliminary screening of the isolates for slime production. The 27 (90%) isolates of *Aeromonas hydrophila* were positive for slime production and 3 (10%) isolates were considered as negative as a result of formation of black consistent crystalline colonies (Figure 1). Then among the 20 isolates of *Aeromonas caviae* 15 (75%) isolates were found to be positive for biofilm production and remaining 5 (25%) considered as negative (Table 3). In this current study, the most of the isolates such as *A.hydrophila* (27), *A.caviae* (15) exhibited Slime production on Congo Red Agar (CRA). It could be concluded that some *Aeromonas* spp. from chlorinated river drinking water sources although are producers of antimicrobial polypeptides, yet they produced virulence factors such as biofilm production when grown under appropriate conditions of growth. Slime comprises of polysaccharides and proteins[24]. Biofilm production involves series of biochemical reactions. It is a complex slime matrix surrounding cells. Biofilm producing bacteria exhibit resistance to more antibiotics than non producers especially plank tonic bacteria. Biofilms aid in surface attachment of microorganisms. This provides conditions favorable for the growth of bacteria. Existence of biofilm producing *Aeromonas* spp. poses a serious danger to public health especially to riverside dwellers. Biofilm extracellular polysaccharide (EPS) matrix serves as barrier to incoming antimicrobial agents. According to the biofilm formation involves cell mobility; attachment to either biomaterial or non biomaterial surfaces and maturation of the cells and thereby producing extracellular matrix that protects the micro colonies from environmental influences.[25] Evaluation of biofilm formation among *Aeromonas* spp. isolated from drinking water and inactivation of biofilm producing isolates by using silver nitrate among the isolates was investigated.

Table 3 Biofilm production

Biofilm production on Congo Red Agar				
Isolates	**Positive**	**%**	**Negative**	**%**
Aeromonas hydrophila (30)	27	90	3	10
Aeromonas caviae (20)	15	75	5	25

Fig. 1.Invitro demonstration of biofilm production on CRA medium

Biofilm positive- Black consistent crystalline colonies, Negative – Colourless colonies

Antibactericidal activity of Silver nitrate on slime/biofilm producing isolates

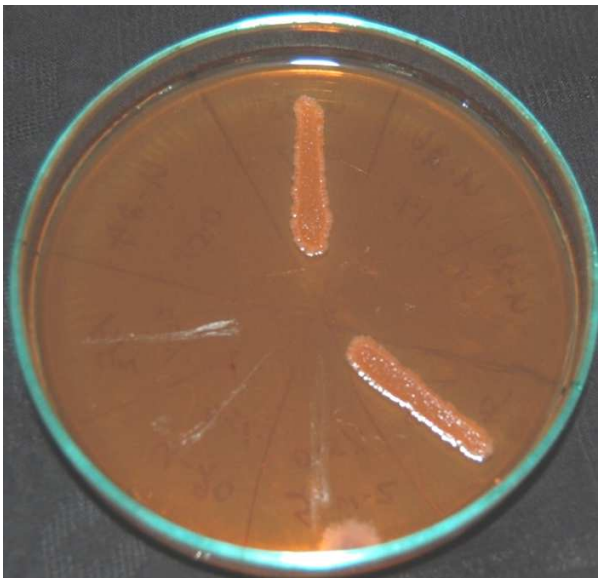
The advantage of silver as a secondary disinfectant in water distribution systems is that it does not form harmful by-products[26]. In the present study, there is no growth of biofilm forming isolates on congo red

agar indicated that *A. hydrophila and A.caviae* were strongly inactivated by silver nitrate in drinking water (Figure 2). The 93% of biofilm producing *Aeromonas hydrophila*(28) were inactivated by silver nitrate and 2(75%) isolates were showed resistant to silver nitrate. In *Aeromonas caviae* (20), 17(85%) isolates were showed positive and 3(15%) isolates showed resistant (Table 4). Although silver appears to be useful in the inactivation of *P. aeruginosa* and *A. hydrophila*, additional work is necessary to demonstrate its potential as a secondary disinfectant, its potential for controlling biofilms and also to study the development of resistance by bacteria in distribution systems over time[27]. In addition, little is known about the ability of *A.hydrophila* to develop tolerance or resistance to silver nitrate. Further studies on the effects of silver on other microorganisms would also be useful. Based on the results of this study and the successful use of silver and copper in large building distribution systems for long durations, silver shows promise as a secondary disinfectant in water distribution systems. The alternation between chlorine and silver may also prove useful in preventing the development of resistance. It could possibly be utilized as a biostatic agent alone or in combination with another disinfectant. Silver resistant bacteria are usually found in areas where bacteria are regularly exposed to silver such as in hospital burn wards, hospital water distribution systems and in contaminated soil near silver mines[28].

Table 4 Silver nitrate (as AgNO3) at 37°C effect on biofilm producing *Aeromonas* isolates

Silver nitrate effect on biofilm formation				
Isolates	**Positive**	**%**	**Negative**	**%**
Aeromonas hydrophila (30)	28	93	2	7
Aeromonas caviae (20)	17	85	3	15

Fig.2. Invitro effect of Silver nitrate on Biofilm producers



Positive→No growth of isolates, Negative→Growth of colonies resistant to silver nitrate

CONCLUSIONS

This study has shown that silver (at a concentration within the secondary standards for drinking water) will inactivate biofilm forming heterotrophic opportunistic pathogenic bacteria found in drinking water distribution systems. This was true even in the presence of humic acid at concentrations that would neutralize the chlorine levels usually found in drinking water. Further research needs to be undertaken in order for silver to be accepted as a disinfectant in certain applications by regulatory agencies. This research should provide sufficient information to corroborate real world observations about the efficacy of silver as a disinfectant and any potential problems related to its use such as the development of microbial resistance.

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