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Original Research Article

Detection of Biofilm-Producing Isolates of *Aeromonas* species from Drinking Water Sources in Ezza South Local Government Area of Ebonyi State, Nigeria

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ABSTRACT

The aim of this study was to screen for biofilm-producing *Aeromonas* species from chlorinated and non-chlorinated drinking water sources. *Aeromonas* species was isolated from the water sources using *Aeromonas* selective medium supplemented with ampicillin and other microbiological media. Susceptibility studies were carried out based on the Kirby-Bauer disk diffusion method; and the inhibitory effect of silver nitrate was also evaluated on the isolated *Aeromonas* species using standard microbiological techniques. A total of 60 isolates of *Aeromonas* species were isolated from both chlorinated and non-chlorinated drinking water samples; and these were positive for biofilm formation. Imipenem, meropenem, ertapenem, cefoxitin, ceftriaxone and cefoxitin were most effective against the isolated *Aeromonas* species. However, ciprofloxacin, ofloxacin, ceftazidime, sulphamethoxazole-trimethoprim, amikacin and ampicillin showed varying rates of susceptibility and resistance to the tested organism. Silver nitrate showed satisfactory inhibitory effect against the biofilm-forming *Aeromonas* species as over 50 % of the isolated *Aeromonas* species were successfully inhibited by the agent. This study reports the presence of biofilm-forming *Aeromonas* species in both chlorinated and non-chlorinated drinking sources in Abakaliki**,** Nigeria; and these organisms are susceptible to silver-nitrate concentrate. Though the usage of silver as a water disinfectant may be subject to water purification regulations, further research is required to quantify and drive the use of this agent in the purification of water – as a panacea to the containment of biofilm-forming microbes in water systems.

Keyword: *Aeromonas* species; water disinfection; antibiotic resistance; biofilms; silver nitrate

INTRODUCTION

The importance of detection of *Aeromonas* species has increased due to their emergent human pathogenic properties [1]. Mesophilic *Aeromonas* spp. is a common organism in the

environment especially in water and sewage, and it also occur in untreated and treated drinking water. Though neither the sources nor the routes of infection with *Aeromonas* species are known, the organism is most often isolated from man in summer when bacillary population becomes the highest in aquatic habitats [2]. *Aeromonas* species have different adhesive abilities, depending on the environmental conditions it is habituating. A single polar flagellum facilitates both adhesion and invasion of human and fish cell lines [3]. In viscous environments or over surfaces, *Aeromonads* are able to produce many peritrichous lateral flagella, which increase bacterial adhesion, and they are also required for biofilm formation in their environment [4]. Studies have also suggested that the non-polar polysaccharide adhesins of *Aeromonads* also play an important role in the adhesion of *Aeromonas* spp to a variety of surfaces [5]. The World Health Organization (WHO) lists *Aeromonas hydrophila* (a common member of the genus *Aeromonas*) as a potential waterborne pathogen. In humans, *Aeromonas* species causes diarrhea, gastroenteritis and extra-enteric conditions such as septicemia, wound infection, endocarditis, meningitis, peritonitis or otitis media. The presence of this organism in drinking water supply including chlorinated water is of public health importance due to their capacity to produce harmful toxins and thus contribute to biofilm development in water distribution systems [6,7]. The number of cases of *Aeromonas* - associated gastroenteritis increases during the summer, and this correlates with an increased number of *Aeromonads* in water systems [8,9]. There is an increasing level of antibiotic resistance among *Aeromonas* species [10]. And they can receive and transfer antibiotic resistance genes to other Gram-negative bacteria in their environment. Biofilms are group of microbes in which cells stick together on a surface which are frequently embedded within a self-produced matrix of extracellular polymeric substance or exo-polysaccharide. And they are important in human infections that are persistent and difficult to treat. The advantage of Silver as a residual disinfectant in water distribution systems is that it does not form harmful by-products [11]. Biofilm producing bacteria including *Aeromonas* species exhibit resistance to more antibiotics than non-producers, and biofilm formation aid in the surface attachment of microorganisms which provides conditions favorable for the growth of bacteria. Thus, this study studied the effect of silver nitrate on the biofilm producing potential of *Aeromonas* species isolated from drinking water sources in Ezza south local government area of Ebonyi State, Nigeria.

MATERIALS AND METHODS

Sample collection

A total of 50 chlorinated and 50 non-chlorinated water samples were collected from different drinking water sources between the months of October 2014 to May, 2015 in Ezza South LGA of Ebonyi State. The samples were collected according to the American Public Health Association (APHA) standard methods in sterile 500 ml of disposable bottles. In order to inactivate chlorine, sterile sodium thiosulphate solution was added (13.2 mg/L) to the chlorinated water. The samples were immediately transported to Applied Microbiology Laboratory, Ebonyi State University, Abakaliki for microbiological analysis. The chlorinated water collected for the study included sachet water, bottled water and tap water while the non-chlorinated water included water from boreholes, streams, pond and well water.

Isolation of *Aeromonas* species

Ten milliliter of Water was inoculated in 100 ml of Trypton Soy Broth (Oxoid, UK) and incubated at 37^{o}C for 24 - 48 hours. A suspension of the turbid medium was streaked on MacConkey

Agar (Micromedia Ltd, India) and incubated at 37^{o}C for 24 - 48 hours and was observed for colony growth [12]. The selected colony was collected with sterile inoculation loop and streaked on nutrient agar (Sigma, Switzerland) for further purification and isolation of pure culture of *Aeromonas* species. Pure Colonies of presumptive isolates were observed morphologically and Gram stained; and the isolates were tested biochemically using motility test, catalase, citrate, indole, voges-proskauer (VP) and oxidase tests. *Aeromonas* selective medium supplemented with ampicillin was used to confirm the isolated *Aeromonas* species.

Screening for the biofilm potential of *Aeromonas* species by tube method

The tube method was performed in two different ways as was described previously [13]. In the first experiment the cultures of *Aeromonas* species were inoculated into 3-5 ml Tryptone soya broth (TSB) (Oxoid, UK) and incubated at 37^{o}C for 48 hours. After incubation, the biofilm formation was observed at the air-liquid interface of the tubes. In the second experiment, cultures of *Aeromonas* species were inoculated into 3-5 ml TSB tubes and incubated at 37^{o}C for 48 hours. After incubation, the content of the tubes were decanted and the tubes were washed with phosphate buffer saline (PBS; pH 7) and left to air-dry. Subsequently, the tubes were stained with crystal violet (0 – 1% w/v), and the tubes were gently rotated to ensure uniform staining. After wards, the stain was removed and tubes were washed with sterile distilled water and then dried in inverted position. Biofilm formation was considered positive when a visible stained film is observed to adhere to the wall and bottom of the tube. Tubes were scored as: 0 = absent, 1 = weak, 2 = moderate or 3 = strong. Experiments were performed in triplicate and results compared with each other for clarity.

Antimicrobial susceptibility tests

The antibiogram of isolated biofilm and non-biofilm producing *Aeromonas* species was determined by the disk diffusion method as described by the National committee for Clinical Laboratory Standard (NCCLS), now Clinical Laboratory Standard Institute (CLSI) [14]. The antibiotic disks used include: ciprofloxacin (5 µg), meropenem (10 µg), ofloxacin (5 µg), tetracycline (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), sulphamethoxazole-trimethoprim (25 µg), ertapenem (10 µg), ceftriaxone (30 µg), amoxycillin (10 µg), chloramphenicol (30 µg), cefoxitin (30 µg), imipenem (10 µg), ampicillin (10 µg), and amikacin (30 µg); and they were procured from Oxoid (Oxoid, UK). Inhibition zone diameters (IZDs) were measured to the nearest millimeter, and breakpoints were defined by NCCLS for Gram-negative bacteria. *A. hydrophila* (ATCC 7966), *A. caviae* (ATCC 15468) were used as controls.

Determination of the inhibitory effect of silver nitrate on *Aeromonas* species with biofilm formation potential

The inhibition of the biofilm production of *Aeromonas* species by silver nitrate was determined by adding 0.2 ml of already prepared silver nitrate solution to 10 ml of TSB. Then the biofilm producing strains of *Aeromonas* species were inoculated into the above solution and incubated at 37^{o}C for 24 hrs. After incubation, the test organism were screened again for the biofilm production using the tube methods as explained above and was observed for the presence or absence of biofilm production after the silver nitrate treatment [15].

RESULTS

A total of 60 isolates of *Aeromonas* species were used for this present day study. The isolates were isolated from 50 chlorinated and 50 non-chlorinated drinking water samples

using standard microbiology techniques for the characterization of *Aeromonas* species. The results of the biochemical/morphological characterization of the *Aeromonas* species are shown in Table 1. Forty isolates were presumptively identified phenotypically as *Aeromonas hydrophila* (66.66%) while 20 isolates were phenotypically identified as *Aeromonas caviae* (33.33%).

Table 2 shows the distribution of *Aeromonas* species isolated from both chlorinated and non-chlorinated water sources. The formation of biofilm by the *Aeromonas* species varies amongst *A. hydrophila* and *A. caviae* in this study (Table 2).

Table 1. Morphological and biochemical characterization of *Aeromonas* species

S/No	Biochemical/morphological Tests	*A. hydrophila*	*A. caviae*
1	Gram staining	Gram negative	Gram negative
2	Motility test	Motile	Non-motile
3	Catalase test	+	+
4	Citrate test	+	+
5	Oxidase test	+	+
6	Indole test	+	+
7	Voges proskauer (VP) test	+	+
	Total number of *Aeromonas* species	40	20

Table 2. Distribution of *Aeromonas* species based on their source and biofilm production

Organism	Chlorinated water n(%)	Non-chlorinated water n(%)	Biofilm production	
			Biofilm Producers	**Non-biofilm producers**
Aeromonas hydrophila	15 (37.5)	25 (62.5)	30	10
Aeromonas caviae	8 (40)	10 (60)	15	5

n=number of isolates

Biofilm production was highest in *A. hydrophila* compared to *A. caviae* as shown in Table 2.

Table 3 shows the results of the antimicrobial susceptibility patterns of the *Aeromonas* species to some commonly used antibiotics. The susceptibility of *A. hydrophila* and *A. caviae* to the tested antibacterial drugs varies.

However, the *Aeromonas* species were more susceptible to the carbapenems including imipenem (100 %), meropenem (100 %), ertapenem (100 %), cefotaxime (100 %), cefoxitin (100 %) and ceftriaxone (100 %). Though the *Aeromonas* species showed some level of susceptibility to ampicillin, ceftazidime,

amoxycillin, and amikacin, some *Aeromonas* species also exhibited varying levels of resistance to the these drugs. High levels of resistance were recorded in sulphamethoxazole/trimethoprim (95 %), chloramphenicol (91.7 %), ofloxacin (83.3 %), ciprofloxacin (83.3 %) and tetracycline (83.3 %). The results of the inhibitory effect of silver nitrate against the biofilm-forming *Aeromonas* species are shown in Table 4. The silver nitrate solution had inhibitory effect of the biofilm-producing *Aeromonas* species. Overall, 60 % of *A. hydrophila* and 17 % of *A. caviae* were successfully inhibited or inactivated by the silver nitrate solution. However, 6.7 % of *A. hydrophila* and 5 % of *A. caviae* were resistant to the antibacterial activity of silver nitrate solution (Table 4).

Table 3. Antimicrobial susceptibility test for *Aeromonas* species

Antibiotics	Susceptible no(%)	Resistant n(%)
Ampicillin	45 (75)	15 (25)
Ceftriaxone	60 (100)	0 (0)
Cefotaxime	60 (100)	0 (0)
Cefoxitin	60 (100)	0 (0)
Ceftazidime	28 (46.7)	32 (53.3)
Amoxycillin	38 (63.3)	22 (36.7)
Ciprofloxacin	10 (16.7)	50 (83.3)
Amikacin	15 (25)	45 (75)
Ofloxacin	10 (16.7)	50 (83.3)
SXT	3 (5)	57 (95)
Tetracycline	10 (16.7)	50 (83.3)
Chloramphenicol	5 (8.3)	5 (91.7)
Imipenem	60 (100)	0 (0)
Ertapenem	60 (100)	0 (0)
Meropenem	60 (100)	0 (0)

SXT=sulphamethoxazole/trimethoprim
n=number of isolates

Table 4. Effect of silver nitrate on biofilm forming *Aeromonas* species

Organism	Positive n(%) (Visible effect)	Negative n(%) (No visible effect)
Aeromonas hydrophila	36 (60)	4 (6.7)
Aeromonas caviae	17 (28.3)	3 (5)

n=number of isolates

DISCUSSION

Water (whether chlorinated or non-chlorinated) serves a variety of purpose in the ecosystem; and this important aspect of life can serve as route via which waterborne pathogens can be transmitted or spread in human populations.

The aquatic environment of Ezza south local government area of Ebonyi state, Nigeria which supports and acts as drinking water sources of indigent people of this locality is often polluted by effluents from various sources including effluents from the markets and other effluent-producing endeavours. *Aeromonas* species including *A. hydrophila* and *A. caviae* are important opportunistic pathogens with the potential of forming biofilms; and their presence in drinking water portend danger to the health of the individuals that patronize such water sources. In this present day study, the biofilm formation potential of *Aeromonas* species was investigated, and the antimicrobial susceptibility patterns of these organisms were also examined. *A. hydrophila* and *A. caviae* were the species of *Aeromonas* isolated from the chlorinated and non-chlorinated drinking water used in this study. *Aeromonas* species as shown in this study are amongst the common bacterial isolates recovered from water sources especially drinking water [4,7,9]. Sixty (60) isolates of *Aeromonas* species were isolated from both chlorinated and non-chlorinated drinking water samples in our study. *Aeromonas* species are water-borne pathogens that have been implicated in several bacterial-related diseases including gastroenteritis [2]. This study shows that *Aeromonas* species is present in both chlorinated and non-chlorinated water. A total of 23 *Aeromonas* species was isolated from chlorinated water while 35 isolates of *Aeromonas* species was recovered from non-chlorinated water sources; and the *Aeromonas* species isolated from these drinking water sources were all biofilm-forming. This finding is similar to a recent study conducted in South Africa – which showed that biofilm-forming *Aeromonas* species inclusive of other bacteria are present in both surface and drinking water distribution systems [16]. The presence of *Aeromonas* species in chlorinated water (aside non-chlorinated water) as shown in this study is an indication that chlorine has little or no effect in inactivating the biofilm-forming ability of *Aeromonas* species. *Aeromonas* species including *A. hydrophila* and *A. caviae* have the ability to grow in water ways and water distribution systems or pipes, and there growth in such aquatic environment is usually characterized by the formation of biofilms (microbial resistant forms) which allows them to withstand the chlorination potentials of water disinfectants. The usage of water contaminated by biofilm-producing *Aeromonas* species could be a source of human infection if such contaminated water is used for human consumption and other domestic purposes. Most of the *Aeromonas* species were highly susceptible to some of the tested antibacterial agents used in this study especially to imipenem, meropenem, ertapenem, cefotaxime, ceftriaxone and cefoxitin. Nevertheless, the *Aeromonas* species showed varying levels of resistance to ciprofloxacin, sulphamethoxazole-trimethoprim, ofloxacin, amikacin and ceftazidime amongst others. In this study, over 50 % of the isolated *Aeromonas* species were successfully inhibited by the silver nitrate solution used. Previous studies have shown that silver nitrate does not form any harmful by-product in water when they are included as a disinfectant in water purification systems [11,15,17]. The incorporation of silver nitrate as a disinfectant (in minimal and tolerable concentration) into water purification systems will help to contain the development and spread of biofilm-producing *Aeromonas* species through water distribution. The use of silver nanoparticles as antimicrobial coatings in water distribution systems is well documented [15]; and this practice shows potential in curbing the problem of development of biofilm-forming microbes inclusive of *Aeromonas* species (as reported in our study) in water distribution pipes or systems.

CONCLUSION

In conclusion, our study have shown that biofilm-producing *Aeromonas* species are present in both chlorinated and non-chlorinated water; and that silver nitrate could be used as a disinfectant in water purification systems. Since chlorine has little effect on the biofilm-formation potential of microbes found in chlorinated water, it is vital to include silver and other chemicals at tolerable concentrations in the treatment of water meant for human consumption – in order to prevent the spread of water-borne disease through this medium.

CONFLICT OF INTEREST STATEMENT

None Declared

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