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ISOLATION OF *Aeromonas* BACTERIOPHAGE AvF07 FROM FISH AND ITS APPLICATION FOR BIOLOGICAL CONTROL OF MULTIDRUG RESISTANT LOCAL *Aeromonas veronii* AFs_2

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ABSTRACT: *Aeromonas* isolates from Nile tilapia fish, fish ponds and River water were identified as well as their bacteriophage specific. Also evaluation of antibacterial effect of both nanoparticles and phage therapy against the pathogenic *Aeromonas veronii* AFs_2. Differentiation of *Aeromonas* spp. was done on the basis of 25 different biochemical tests and confirmed by sequencing of 16s rRNA gene as (*A. caviae* AFg, *A. encheleia* AWz, *A. molluscorum* AFm, *A. salmonicida* AWh, *A. veronii* AFs_2, *A. veronii bv. veronii* AFi). All of the six *Aeromonas* strains were resistant to β-actam (amoxicillin/ lavulanic acid) antibiotics. However, the resistance to other antibiotics was variable. All *Aeromonas* strains were found to be resistant to ampicillin, cephalexin, cephradine, amoxicillin/clavulanic acid, rifampin and cephalothin. Sensitivity of 6 *Aeromonas* strains raised against 7 concentrations of chitosan nanoparticles. Using well diffusion method spherically shaped silver nanoparticles AgNPs with an average size of ~ 20 nm, showed a great antimicrobial activity against *A. veronii* AFs_2 and five more strains of *Aeromonas* spp. At the concentration of 20, 24, 32 and 40 µg/ml. Thermal inactivation point was 84°C for phage AvF07 which was sensitive to storage at 4°C compared with the storage at -20°C. Intraperitoneal injection in fish using phage AvF07 together with *A. veronii* AFs_2, no mortality was shown until the end of experiment (14 days). However, mortality of 43.8% or 50% was obtained after 2 or 3 days, respectively, when chloramphenicol was injected instead of phage.

Key words: *Aeromonas veronii*, *Aeromonas* phage, phage therapy, AgNPs, chitosan.

INTRODUCTION

Aeromonas species are facultatively anaerobic Gram negative bacterium that belongs to the family *Aeromonadaceae*. Aeromonads are primarily inhabitants of the aquatic environment including ground water, lakes, drinking water and wastewater. Humans acquire this organism from a wide range of food and water sources as well as during aquatic recreational activities **(Tomás, 2012; Khor *et al*., 2015; Bello *et al*., 2016)**. Thus the genus *Aeromonas* is considered as an emerging pathogen and identified as a high-risk carrier **(Reshma *et al*., 2015)**.

It is very important to combat *Aeromonas* because of its growing importance as an emerging pathogen. *Aeromonas* strains may produce many different putative virulence factors, (enterotoxins, hemolysins or cytotoxins) **(Kumar *et al*., 2015)**. Also, Fish and chicken play an important role in the transmission of this pathogen to humans **(Praveen *et al*., 2016)**.

The global rise in antimicrobial resistance (AMR) among bacteria causing infectious diseases is well documented, and the associated risks for human health are well known. *Aeromonas* is widely distributed in the environment and causes many diseases in fish and humans **(Piotrowska and Popowska, 2014)**.

As potential antimicrobial agents, phage therapy in animal production is a renewed interest on the application of bacteriophages. Phage research continues to open new approach in using phages in the stages of "farm to fork" **(Boari *et al*., 2008)**.

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The concept of phage therapy has been revisited and expanded upon over the past twenty years for the elimination of pathogenic bacteria **(Keary *et al*., 2013)**.

Consumption of beyond-accept- able-limit of antibiotic residuals can affect people's health and well-being in the long term. In addition, improper usage of antibiotics can greatly affect the long term sustainability of the aqua industry in general, and striped catfish sector in particular, due to the negative biological impact within the farming environment and over time the eco system at large. Due to these adverse impacts of *A. hydrophila* in aqua-culture, there is an urgent need to come up with an alternative solution, effectively and eco-friendly such as phage therapy **(Hoang *et al*., 2019)**. Therefore, the aims of the present study were: Isolation and identification of *Aeromonas* spp. as well as its bacteriophage specific. Also evaluation of antibacterial effect of nanoparticles and phage therapy against the pathogenic *Aeromonas veronii* AFs_2.

MATERIALS AND METHODS

Nile Tilapia Fish (*Oreochromis niloticus*)

Fish samples were purchased from local fish market at Zagazig City, Sharkia Governorate, Egypt, and transferred quickly in sterilized plastic bags to the Laboratory of Agricultural Microbiology Department, Faculty of Agriculture, Zagazig University, Egypt, for isolating *Aeromonas* spp. as well as counting the total bacterial counts during the period between June 2014 and May 2015.

Isolation of Putative *Aermonas* Isolates

Ten fish samples were collected each time during one year of experiment for isolation process. The different samples included muscles, skin, gills and intestinal tissues for isolation **(Yadav *et al*., 2014)** and counting *Aeromonas* spp. The samples were homogenized in peptone water (PW) using a stomacher blender. The appropriate dilutions were inoculated onto plate count agar (PCA) (Components g/L: Pancreatic digest of casein 5.0 **,** Yeast extract 2.5, Glucose 1.0, Agar 15.0, Distilled water up to 1000ml, pH 7.0 ± 0.2 seen in **Atlas (2004)** or LAB167 *Aeromonas* Agar Bile Salt Irgasan Brilliant Green Agar (Components g/L: Beef extract 5.0, Meat peptone 5.0, Xylose 10.0, Bile saltes NO.3 8.5, Sodium thiosulphate 5.44, Iragasan 0.005, Brilliant green 0.005, Neutral red 0.025, Agar 11.5, Distilled water up to 1000 ml, pH 7.0 ± 0.2 seen in **Corry *et al*. (2003**) and incubated at 30°C or 37°C for 48 hr., or 24 hr., respectively.

Aeromonas spp. Counts

A volume of 100 µl of appropriate dilutions and spread evenly over the surface of each *Aeromonas* selective agar base **(Yadav *et al*., 2014)**, plate with a sterile bent glass rod, and incubated at 37°C for 24 hr. *Aeromonas* spp. were represented by presumptive green with darker (mostly black) centered colonies surrounded by clear zones and yellow to honey color.

Identification of Six *Aeromonas* Isolates

After being isolated and purified, putative *Aeromonas* isolates were subjected to 25 biochemical tests as recommended in the Bergey's Manual of Systematic Bacteriology 2nd edition, volume two, (The proteobacteria) part B, The Gamma Proteobacteria by **Martin-Carnahan and Joseph (2005)** and confirmed using 16SrRNA in Sigma Scientific Services Company, Giza, Egypt according to the protocol of **Maniatis *et al*. (1989),** and the GeneJet genomic DNA purification Kit (Thermo K0721).

Antibiotics Susceptibility Test

Antibiotic susceptibility tests on 22 antibiotics were performed by the standard disc diffusion method **(NCCLS, 2003 and 2004)**. Results were recorded as resistant, intermediate and sensitive after measuring the diameter of the inhibition zones (mm) and compared with the standards for antimicrobial disk susceptibility tests, using Luria Bertani (LB) media Laboratories, **(Belém-Costa and Cyrino, 2006; Furmanek-Blaszk, 2014)**. Zone diameter breakpoints for *Aeromonas* was assessed according to the Committee for Antibiogram of the French Society of Microbiology 2010 as seen in **Lamy *et al*. (2012) and Samal *et al*. (2014)**.

Antibacterial Activity of Silver and Chitosan Nanoparticles on *Aeromonas veronii* AFs_2

Antibacterial activity of spherical silver nanoparticles (AgNPs), of ~ 20 nm diameter at 20, 24, 32 and 40 µg/ml concentrations, were

determined using agar well diffusion assay **(Sarkar *et al*., 2012; Qais *et al*., 2019)**. Silver nanoparticles (AgNPs) were kindly obtained from Nanotech Gate 3, Dreamland, 6th October, Cairo-Egypt, http://www.nanotecheg.com.

While the antibacterial activity of chitosan nanoparticles with spherical shape of ~ 150 nm at seven concentrations namely (2.0, 1.0, 0.5, 0.25, 0.12, 0.06 and 0.03 µg/ml) were determined using the agar disk diffusion assay **Qi *et al*. (2004)**. Chitosan nanoparticles were kindly obtained from Nanotech Gate 3, Dreamland, 6th October, Cairo-Egypt, http:// www.nanotecheg.com.

Isolation of Phage AvF07

Phage isolation was conducted as described by **Wommack *et al*. (2009) and Hyman (2019).** Twenty five grams of fish samples were mixed with 225 ml of peptone saline water (PSW). After shaking for three hours at 250 rpm, centrifugation at a low speed 4000 rpm for 20 min. was done using Hettich Zentrifugen D-78532, made in Germany to precipitate debris . The supernatants were filtered through 0.45µm membrane filters (Gelman Science, Inc., Ann Arbor, Mich) to exclude bacterial debris. *Aeromans veronii* AFs_2 strain was used as potential recipient to detect the possible presence of *Aeromonas veronii* AFs_2 phage.

Phage Purification and Host Specificity

A single plaque was purified by five successive single passages as described by **Eisenstark (1967) and Kabanova *et al*. (2019)** with susceptible *Aeromonas veronii* AFs_2 and the final single plaque strain was designated as AvF07. The supernatant was kept in the refrigerator at 4°C. The host range of the phage was tested by spot test **(Ackermann *et al*., 1978)** experiments at a titer of 10^{5-6} PFU/ml.

Electron Microscopy

Potassium phosphotungstate 1% (PTA at pH 7.0) was used for negatively staining of phage suspension. Images of stained phage samples on carbon-coated, 400-mesh copper grids were captured by CCD camera model AMT, optronics, with 1632x1632 pixel formate as side mount configuration. This work was done in TEM lab in FARP JEM 1400 (Faculty of Agriculture Research Park-Cairo University). Based on their morphology, phages were identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses **(Fauquet *et al*., 2005)**.

Phage Sensitivity to Some Physical Factors

Temperature sensitivity

An optimal phage dilution was prepared using LB medium **(Atlas, 2004)**. The diluted tubes were placed in water bath at 50, 60, 70,80 and 84°C for 10, 30 and 60 min. . Phage titration was assayed for infectivity using plaque assay method **(Adams, 1959)**.

Longavity of phage under storage temperatures

The stability of *A. veronii* phage AvF07 was determined at various storage temperatures. Phage suspension was incubated at ambient temperature (22±2°C), refrigerator (4°C) or at freezer (-20°C) for various periods and samples were withdrawn at different periods. Loss of phage infectivity was assayed using double layer technique **(Adams, 1959).**

pH sensitivity

Survival at different pH values (pH from 1-12) at 37°C was carried out as previously discribed by **Verma *et al*. (2009).**

In vivo antibacterial activity of phages, nanoparticles or antibiotics against *Aeromonas veronii* AFs_2

A total of 272 fish apparently healthy Nile tilapia (*Oreochromis niloticus*) were obtained from Centeral Laboratory for Aquaculture research-Abuohamad-Abassa, Sharkia Governorate, Egypt, and they were grown in glass aquaria measuring 50 x 30x 40 cm. A sum of 16 fish (about 40 ± 3 g) were used in each of two aquaria and each group of fish was intraperitoneal injected with fresh bacterial suspension (2.3 x 10^6 CFU/fish) alone or with phage AvF07 at Multiplicity of infection (MOI) 1 as indicated in the results. Also, no injection or intraperitoneally injection with sterilized distilled water, phage $2.3x10^6$ PFU/fish, silver nanoparticles (AgNPs) 20 µg/fish, chitosan nanoparticles 200 µg/fish, or Chloramphenicol 30 µg/fish were used as a control. Fish were intraperitoneally injected with

bacterial suspension ($2.3x10^6$ CFU/fish) each was separately injected with silver nanoparticles (AgNPs) 20 µg/fish. Fish were intraperitoneally injected with bacterial suspension ($2.3x10^6$ CFU/ fish) each was separately injected with chitosan nanoparticles 0.2µg/fish. Finally, fish were intraperitoneally injected with bacterial suspension ($2.3x10^6$ or $2.3x10^7$ CFU/fish) each was separately injected with chloramphenicol 30 µg/fish.

Observation of abnormalities and counting the mortality of fish were performed every 24 hr., until the end of the experiments (The observation time was 14 days) **(Shayo *et al.*, 2012; El-Araby *et al*., 2016)**.

Statistical Analysis

All data were entered into Excel sheet (2010). The Log_{10} of the mean of three replicates were calculated and standard diviation was measured with Excell program (± SD).

RESULTS AND DISCUSSION

Identification of Six *Aeromonas* spp. using 16S rRNA Sequence

A total of 376 *Aeromonas* spp. were identified on the basis of biochemical tests and six of them were confirmed by sequencing of 16S rDNA gene as (*A. caviae*, *A. encheleia*, *A. molluscorum*, *A. salmonicida*, *A. veronii* and *A. veronii bv. veronii*).

The results for the identification of the aforementioned isolates based on the biochemical tests reported by **Martin-Carnahan and Joseph (2005)** are presented in Table 1. The isolates were identified as *Aeromonas caviae*, *Aeromonas enecheleia*, *Aeromonas molluscorum*, *Aeromonas salmonicida*, *Aeromonas veronii*, *Aeromonas veronii bv. veronii*. Generally, the criteria to identify species were primarily based on biochemical tests then the sequencing of the 16S rDNA gene has proven to be valuable in the identification of *Aeromonas* spp. **(Martínez-Murica *et al.*, 2000)**. The overall sequence similarity between *Aeromonas* spp. was very high, but there was sufficient variability to discriminate different species. PCR-RFLP analysis of 16S rRNA gene was considered to be a rapid and powerful method for identifying isolates of *Aeromonas* to the species level **(Borrell *et al*., 1997; Ghatak *et al*., 2007)**.

The invaluable methods for the identification of *Aeromonas* spp. were PCR amplification and restriction digestion of the16S rRNA. Six isolates were identified in Sigma Scientific Services Company, Giza, Egypt using 16S rRNA as follows: DNA isolation was done according to the protocol of **Maniatis *et al.* (1989)**, and the GeneJet genomic DNA purification Kit (Thermo K0721). A good and rapid way of assessing the identities of all known species of *Aeromonas* is computer analysis of the published 16S rRNA gene **(Borrell *et al*., 1997)**.

The most apparently six different isolates of *Aeromonas* spp. compared with the other *Aeromonas* spp. tested using 16S rRNA are shown in Fig. 1 and Table 2.

The obtained results in this study were similar to those found by **Martínez-Murica *et al.* (2000)** who stated that the genus comprises the species *Aeromonas hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (*biovars sobria* and *ceronii*), *A.jandaei*, *A.schubertii*, *A.trota*, *A. allosaccharophila*, *A. encheleia* and *A. popoffii* and two DNA homology groups, *Aeromonas* spp. HG11 and *Aeromonas* spp. HG13 (formerly enteric group 501), which still without a species name.

Bacterial Strains

Six bacterial strains were isolated and identified in this study namely as *Aeromonas caviae* AFg, *Aeromonas encheleia* AWz, *Aeromonas molluscorum* AFm, *Aeromonas salmonicida* AWh, *Aeromonas veronii* AFs_2 and *Aeromonas veronii bv. veronii* AFi.

Antibacterial Activity of Different Antibiotics Against *Aeromonas* Strains

The resistance patterns of eight *Aeromonas* spp. against 22 antibiotics are given in Table 3. Based on the average inhibition zone for each antibiotic, there was an obvious variation in their sensitivity. Results in Table 3 show that all *Aeromonas* spp. were resistant to ampicillin, cephalexin, cephradine, amoxicillin/clavulanic acid, rifampin as well as to cephalothin. These

Table 1. Biochemical properties of some *Aeromonas* spp. isolated from fish and water samples

Characteristic	Bacterial isolate					
	AFg	AWz	AFm	AWh	AFs_2	AFi
Motility	+	+	+	+	+	+
Indole production	+	+	+	+	+	+
Voges – proskauer	-	-	-	+	-	+
Urea hydrolysis	-	-	-	-	-	-
H_2S production	-	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Acid glucose	+	+	+	+	+	+
Gas glucose	-	+	+	+	+	+
Growth in 0% NaCl	+	+	+	+	+	+
Growth in 3% NaCl	+	+	+	+	+	+
Acid mannitol	+	+	+	+	+	+
Rabinose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-
Rhamnose	-	+	-	-	-	-
Mannose	+	-	+	+	+	+
Ribose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Lactose	+	-	+	+	+	+
Sucrose	+	+	+	+	+	+
Starch	+	+	+	+	+	+
Sorbitol	-	-	-	+	-	-
Trehalose	+	+	+	+	+	+
Cellobiose	+	-	+	+	+	+

AFg: isolated from gills. , AWz: isolated from mowees river., AFm: isolated from muscles. , AWh: isolated from Abou-Hammad irrigation canal. , AFs_2: were isolated from skin., AFi : isolated from intestine.

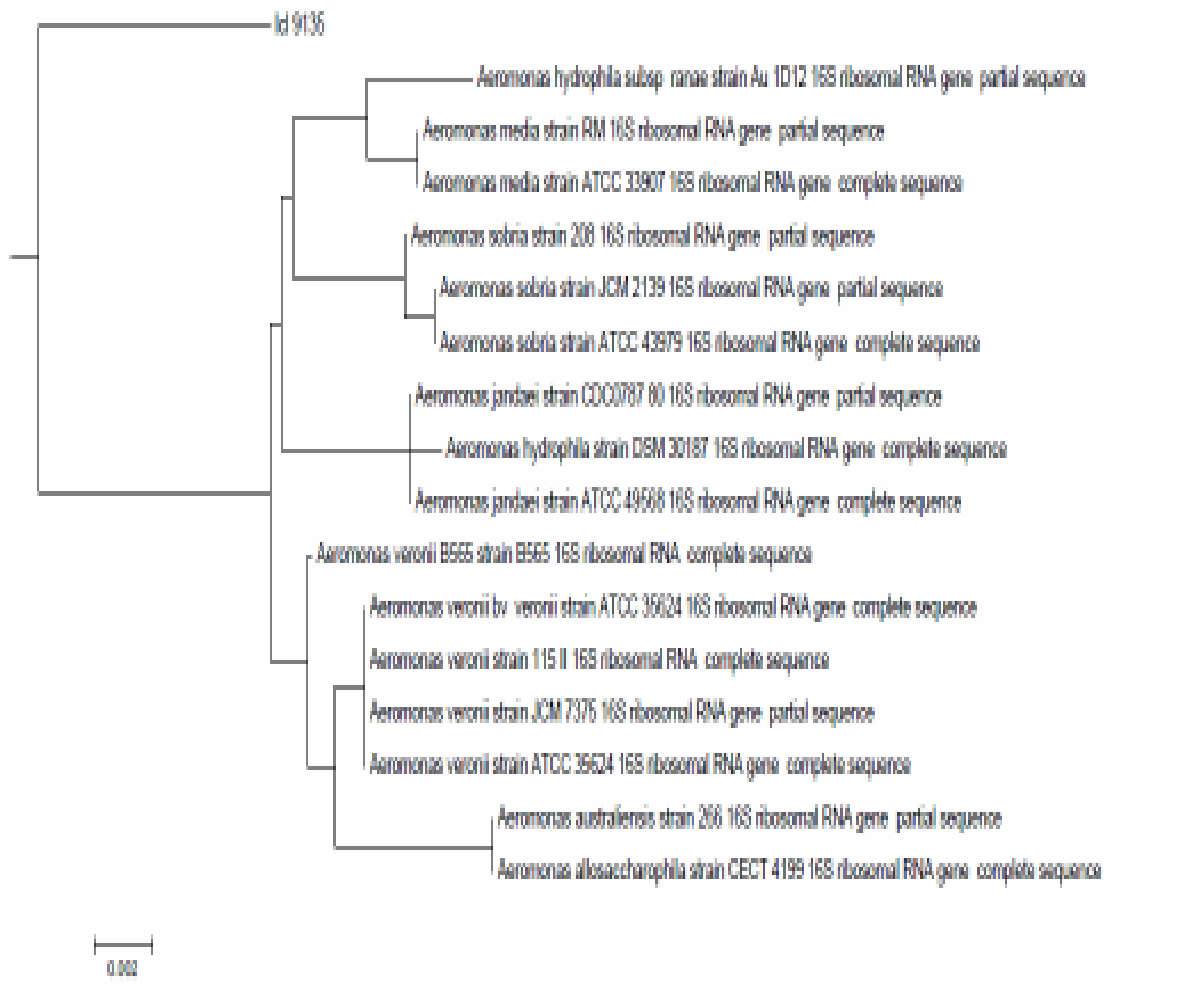


Fig. 1. Phylogenetic tree based on 16S rRNA of *Aeromonas veronii* B565 strain B565 16S ribosomal RNA gene, two *Aeromonas media*, three *A. sobria*, two *A. jandaei* and one species from each of the following: *A. hydrophila*, *A. veronii bv. veronii*, *A. australiensis*, *A. hydrophila* subsp *ranae* and *A. allosaccharophila*

Table 2. Identification of six *Aeromonas* species

Isolate	Identification by biochemical and 16S rRNA	Identity	International *Aeromonas* strains
AFg	*Aeromonas caviae*	98%	*Aeromonas caviae* strain CECT 4221 16S ribosomal RNA gene, partial sequence
AWz	*Aeromonas encheleia*	98%	*Aeromonas encheleia* strain CECT4342 16S ribosomal RNA gene, partial sequence
AFm	*Aeromonas molluscorum*	90%	*Aeromonas molluscorum* strain LMG 22214 16S ribosomal RNA gene, complete sequence
AWh	*Aeromonas salmonicida*	97%	*Aeromonas salmonicida* strain ATCC 33658 16S ribosomal RNA gene, complete sequence
AFs$_2$	*Aeromonas veronii*	98%	*Aeromonas veronii* B565 strain B565 16S ribosomal RNA, complete sequence
AFi	*Aeromonas veronii bv.veronii*	97%	*Aeromonas veronii bv.veronii* strain ATCC 35624 16S ribosomal RNA gene, complete sequence

Table 3. The susceptibility of 6 *Aeromonas* strains to 22 antibiotics, based on the diameter of inhibition zone (mm)

Antibiotic and *Aeromonas* strain MAR	AK (30 µg)		AM (10 µg)		AMC (30 µg)		ATM (10 µg)		AX (25 µg)		C (30 µg)		CE (30 µg)		CIP (5 µg)		CL (30 µg)		CN (10 µg)		DO (30µg)		Index of MAR
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
A.caviae (AFg)	22	S	8	R	0	R	9	R	18	I	23	S	0	R	21	R	0	R	17	I	14	I	0.59
A.encheleia (AWz)	13	R	0	R	0	R	0	R	0	R	16	I	8	R	23	I	0	R	18	S	20	S	0.55
A. molluscorum (AFm)	13	R	0	R	0	R	21	I	11	R	22	S	0	R	26	S	7	R	13	R	18	S	0.64
A.salmonicida (AWh)	16	I	12	R	0	R	0	R	16	I	16	I	0	R	16	R	0	R	16	I	18	S	0.68
A.veronii (AFs_2)	16	I	8	R	0	R	0	R	0	R	21	S	0	R	0	R	0	R	15	R	0	R	0.86
A.veronii.bv. veronii (AFi)	15	I	13	R	7	R	21	I	12	R	21	S	9	R	22	I	8	R	17	I	10	R	0.5
Average	15.8		6.8		1.1		8.5		9.5		19.8		2.8		18		2.5		16		13.3		0.63

AK: Amikacin, AM: Ampicilin, AMC: Amoxicillin/clavulanic acid, ATM: Aztreonam, AX: Amoxicillin, C: Chloramphenicol, CE: Cephradine, CIP: Ciprofloxacin, CL: Cephalexin, CN: Gentamicin, DO: Doxycycline. (1): Inhibition zone (mm), (2): S/R/I: S: sensitive, R: resistant, I: intermediate according to **(Lamy *et al*., 2012; Samal *et al*., 2014)**. MAR: Multiple Antibiotic Resistance Index. MAR Index= Number of antibiotics to which the strain was resistant/Total number of antibiotics tested **(Raja and John, 2015)**.

Table 3. Cont.

Antibiotic *Aeromonas* strain and MAR	E (15µg)		FOX (30 µg)		K (30 µg)		KF (30 µg)		N (30 µg)		NA (30 µg)		NOR (10 µg)		OFX (5 µg)		RA (5 µg)		SXT (1.25/ 23.75µg)		TE (30 µg)		Index of MAR
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
A.caviae (AFg)	7	R	0	R	17	S	0	R	16	I	21	S	18	S	17	R	0	R	7	R	0	R	0.59
A.encheleia (AWz)	7	R	15	I	16	I	0	R	11	R	0	R	17	S	23	I	0	R	11	I	17	I	0.55
A. molluscorum (AFm)	0	R	23	S	12	R	0	R	22	S	0	R	30	S	18	R	10	R	15	I	0	R	0.64
A.salmonicida (AWh)	12	R	11	R	17	S	0	R	12	R	0	R	0	R	15	R	0	R	18	S	0	R	0.68
A.veronii (AFs_2)	0	R	12	R	13	R	0	R	0	R	0	R	0	R	17	R	0	R	11	I	0	R	0.86
A.veronii.bv. veronii (AFi)	14	I	13	R	16	I	0	R	20	S	23	S	25	S	17	R	0	R	13	I	7	R	0.5
Average	6.6		12.3		15.1		0		13.5		7.3		15		17.8		1.6		12.5		4		0.63

E : Erythromycin, FOX: Cefoxitin, K: Kanamycin, KF: Cephalothin, N: Neomycin, NA : Nalidixic acid , NOR : Norfloxacin, OFX : Ofloxacin , RA : Rifampin, SXT: Trimethoprim/sulphamethoxazole, TE : Tetracycline. (1): Inhibition zone (mm), (2): S/R/I: S: sensitive, R: resistant, I: intermediate according to **(Lamy *et al*., 2012; Samal *et al*., 2014)**. MAR: Multiple Antibiotic Resistance Index. MAR Index= Number of antibiotics to which the strain was resistant/Total number of antibiotics tested **(Raja and John, 2015)**.

On the other hand, chloramphenicol was the most active antibiotic against 5 *Aeromonas* strains compared to the others resulted in 19.8 mm as an average inhibition zone followed by ciprofloxacin which resulted in 18 mm (Table 3). These results are in harmony with those of **Belém-Costa** and **Cyrino, (2006)**, since they observed that the *A. hydrophila* type strain presented resistance to the aforementioned antimicrobial substances and also against rifampicin. However, **Laith** and **Najiah (2013)** stated that the majority of *Aeromonas* spp. isolated from diseased fish were *A. hydrophila*. All isolates of *A. hydrophila* were resistant to ampicillin and susceptible to tetracycline. Multiple antibiotic resistance index (MAR) for all isolates ranged between 0.10 to 0.50. Therefore, routine monitoring of drug susceptibility pattern over time is necessary. **Samal *et al.* (2014)** found that *A. hydrophila* isolated from diseased fish were sensitive to oxytetracycline, ofloxacin, azithromycin, doxycycline, nitrofurazone, streptomycin, chlorotetracycline and norfloxacin.

Concerning the number of the sensitive *Aeromonas* strains observed in this study, the following category could be noticed: chloramphenicol (5 strains), norfloxacin (4 strains), doxycycline (3 strains), and each of kanamycin, gentamycin, neomycin and nalidixic acid (2 strains). Similar results were found by **Dias *et al.* (2012)** who reported that all *Aeromonas* spp. (*Aeromonas veronii*, *Aeromonas media*, *Aeromonas jandaei*, *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas culicicola*, and *Aeromonas aquariorum*) were sensitive to cefotaxime and cefepime. Their results showed that those *Aeromonas* spp. strains are potentially considered reservoirs of antibiotic resistance genes.

Multiple antibiotic resistance was observed in *Aeromonas* spp. in our study since *A. veronii* AFs_2 was resistant to 19 antibiotics. Also *A. salmonicida* AWh, *A. molluscorum* AFm and *A. caviae* AFg were resistant to 15, 14 and 13 different antibiotics, respectively. While *A. veronii. bv. veronii* AFs_1 was resistant to 11 antibiotics (Table 3). From these results it could be shown that *A. veronii* AFs_2 recorded the highest MAR indices giving 0.86; respectively. *A. veronii* was highly sensitive to only one antibiotic (chloramphenicol). These results are in harmony with those reported by **Vivekanandhan *et al.* (2002), Raja and John (2015) and Wickramanayake *et al.* (2019)** who found that multiple antibiotic resistance (MAR) has been registered for *A. hydrophila* isolated from freshwater fish farms in association with a variety of drugs, commonly used as feed additives.

Effect of Silver and Chitosan Nanoparticles on *Aermonas veronii* AFs_2

Silver nanoparticles synthesized chemically with an average size of ~ 20 nm and spherical shape and chitosan nanoparticles which were more or less uniform in size and shape Fig. (2.B) and the diameter of 124-177 nm with an average of ~150 nm by scale bar in TEM (Fig. 2) were studied for their antibacterial activities against *A. veronii* using well diffusion assay. Results in Table 4 show the susceptibility of *Aeromonas veronii* AFs_2 to 4 silver nanoparticles (AgNPs) concentrations. *A.veronii* AFs_2 gave halo diffusion measurment 27.5 mm. Similar results were obtained by **Sarkar *et al.* (2012)** who found that the antimicrobial activities of chemically synthesized silver nanoparticles exhibited antimicrobial efficacy in both the standared inhibitory assays (well diffusion method and growth curve analysis).

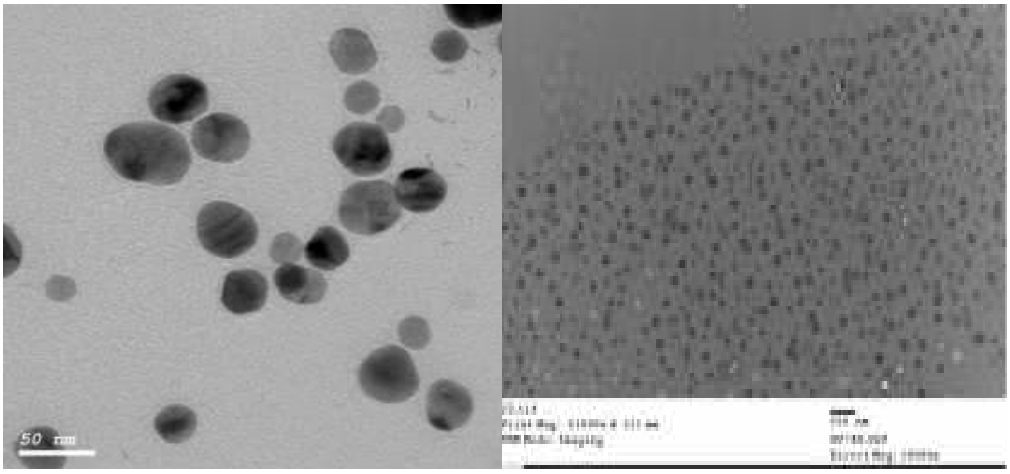
Antibacterial activities using disc diffusion method were shown against *Aeromonas veronii* AFs_2 when seven different concentrations of chitosan nanoparticles were used. Inhibition zone increased with increasing the concentrations of chitosan nanoparticles giving a maximum diameter of 26±3 with *Aeromonas veronii* AFs_2 when 2.0 µg/ml was used. To interprete this inhibitory effect **Qi *et al.* (2004)** stated that exposure of *Salmonella choleraesuis* to the chitosan nanoparticles led to the disruption of cell membranes and the leakage of cytoplasm.

The inhibition zones obtained with the previous *Aeromonas veronii* AFs_2 ranged from 12 up to 26 mm when the concentrations of chitosan nanoparticles were 2.0, 1.0, 0.5, 0.25, 0.12, 0.06 and 0.03µg/ml.

Similar results were obtained by **Ibrahim *et al.* (2015)** who stated that chitosan nanoparticles could inhibit the growth of $Gram^+$ and $Gram^-$

Table 4. Inhibitory effect of different concentrations of silver and chitosan nanoparticles on *Aeromonas veronii* AFs_2

Concentration of nanoparticle (µg/ml)		Inhibition zone (mm)	Average
AgNPs	**20**	21±1	27.5
	24	24±1	
	32	29±1	
	40	36±2	
Chitosan nanoparticles	**0.03**	12±3	19.3
	0.06	15±1	
	0.12	17±2	
	0.25	20±3	
	0.5	21±3	
	1.0	24±2	
	2.0	26±3	



(A) **(B)**

Fig. 2.A. TEM micrograph of chemically synthesized silver nanoparticles, B. TEM image of chitosan nanoparticles

bacteria. **Chávez de Paz *et al.* (2011)** found earlier that low-MW chitosans showed high antimicrobial effect (>95% of cells damaged) against *Streptococcus mutans* biofilms. Also, **Aliasghari *et al.* (2016)** reported that the MIC of chitosan nanoparticle for *S. mutans, S. salivarius* and *S. sobrinus* was 0.625 mg/ml and for *S. sanguis* was 0.312 mg/ml. They added that chitosan and chitosan nanoparticles at a concentration of 5 mg/ml also reduced biofilm formation of *S. mutans* up to 92.5% and 93.4%, respectively.

Isolation of *Aeromonas* Phages and Determination of their Host Range

Three phages were isolated from different sources (*i.e.*, from fish and water) using *A. sobria* AFs_1, *A.veronii* AFs_2 and *A.veronii bv. veronii* AFi as hosts. Then, only one phage was chosen (phage AvF07) to find out its host range and was named according to the newly proposed naming system **(Kropinski *et al.*, 2009; Hyman, 2019)**. Six different *Aeromonas* spp. strains as shown in Table 5 to examine their susceptibility to the

isolated *Aeromonas* phage. Generally, phage AvF07 exhibited the highest lytic activity against *Aeromonas* spp. tested *i.e*., 75%. The other bacterial species gave clear plaques while four bacterial strains gave a turbid plaques. These results are similar to those obtained by **Ahmady (2016)** who mentioned that the environmental 64 bacterial isolates exhibited different patterns of lysis by different phages studied, which reflect heterogenecity in *Enterobacter* spp. and *Aeromonas* spp. populations and genetic diversity amongst the phage isolates. Results in Table 5 show also that there was no relation between the susceptibility of *Aeromonas* spp. to the tested phage and the source of bacteria (fish or water).

Bacteriophage (AvF07) Morphology

The morphological features of phage AvF07 particles isolated from *Aeromonas veronii* AFs_2 colonized Nile tilapia fish are shown in Fig. 3 after negatively stained with 1% potassium phosphotungstate (PTA at pH 7.0). Based on the morphology, phage was identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses. This phage possessed a hexagonal outline head measuring (75 x 75 nm), long noncontractile tail measuring (250 x 15 nm), and base plate with 25 nm width and 18.8 nm height. In addition to few tail spikes attached to the base plate (not shown in Fig. 3). This phage might be tentatively classified under *Siphoviridae*. When the spot test does not appear colony, which indicates that there is no lesogny for this phage and thus be fit for use in the therapy. This phage is different from that previously isolated by **Chow and Rouf (1983)** since they reported that two *A. hydrophila* bacteriophages, Aehl and Aeh2, were composed of a head and contractile tail which might be belong to the morphological group A of bacteriophages described by **Bradley (1967)**.

In contrast, **Megahed (2016)** stated that two *A.hydrophila* phages ϕzH_1 and ϕzH_2 isolated from Nile water consisted of icosahedral heads measured 100 and 50 nm with very short tail measured 30 and 7 nm, respectively. Accordingly those phages belong to the family *Podoviridae*.

Sensitivity of *Aeromonas veronii* AFs_2 Phage AvF07 to Various Physical Factors

Thermal inactivation

Heat resistance capability of phage AvF07 was performed for 10, 30 and 60 min. The results showed that AvF07 was extremely heat stable at 50°C (Fig. 4), hence the survival percent was 98.7, 96.2 and 82.3 after 10, 30 and 60 min., respectively. Similar results were obtained by **Han *et al.* (2014)** who reported that φPA-HF17 was extremely heat stable; ~ 100% phage particles (10^7 PFU/ml) survived for 30 min and 60 min at 50°C. They also found that the number of viable phages decreased from 1×10^7 PFU/ml to 1×10^6 PFU/ml and 4.5×10^6 PFU/ml after 30 min and 60 min at 60°C, respectively. However exposure to 70 °C for 60 min. inhibited activity by 60.8%, while exposure to 80°C, a reduction of 39.2% was recorded after 10 min. but a reduction of virus particles reached 100%, for 30 min or an hour.

These results are comparable with those obtained by **Mishra *et al.* (2012)** who reported that phage F20 (*E. aerogenes*) survived at 70°C for 150 min with only a slight reduction in titre (<0.2 log 10 PFU/ml^{-1}). By contrast, *A. hydrophila* phages ϕzH_1 and ϕzH_2 of *Siphoviridae* were thermal sensitive and completely inactivated at 70°C **(Megahed, 2016)**. They concluded that a *Siphoviridae* phage AvF07will be considered as a thermostable phage. Similar reports were presented by **Lu *et al.* (2002), Lin *et al.* (2010) and Fan *et al.* (2019)** who stated that a comparatively, thermostable phage from the family *Siphoviridae* was stable at 50°C but their titers were dramatically decreased by 50% at 80°C.

Storage temperatures

Phage under this investigation was maintained at ambient temperature (22± 2°C), 4°C or at -20°C for a time as indicated. Concerning the ambient temperature, the highest reduction 100% in phage infectivity was observed after storage for more than 14 days. So the time of incubation is considered an important factor since the reduction of phage AvF07 was found 11.2 – 71.2% after 1 and 14 days of storage, respectively. The results contradict those obtained by **Mishra *et al.* (2012)** who found a good stability at 25°C over 6 months with phage F20 (*E. aerogenes*) with only a slight decrease in titre and survived maintenance at 4°C up to 4 months.

Table 5. Susceptibility of *Aeromonas* spp. to the selected phage under this study

***Aeromonas* strain**	**Bacterial Source**	**Phage***
		AvF07
***Aeromonas caviae* AFg**	Fish	-
***Aeromonas encheleia* AWz**	Surfac water	$^{+}$t
***Aeromonas molluscorum* AFm**	Fish	$^{+}$t
***Aeromonas salmonicida* AWh**	Surfac water	$^{+}$c
***Aeromonas veronii* AFs_2**	Fish	$^{+}$c
***Aeromonas veronii bv. veronii* AFi**	Fish	$^{+}$c

* Phage key : AvF07 wase isolated from fish . - = not formed lytic area; $^{+}$t = gave lytic area and formed plaque after plaque assied; +c = clear plaque; t, turbid plaque.

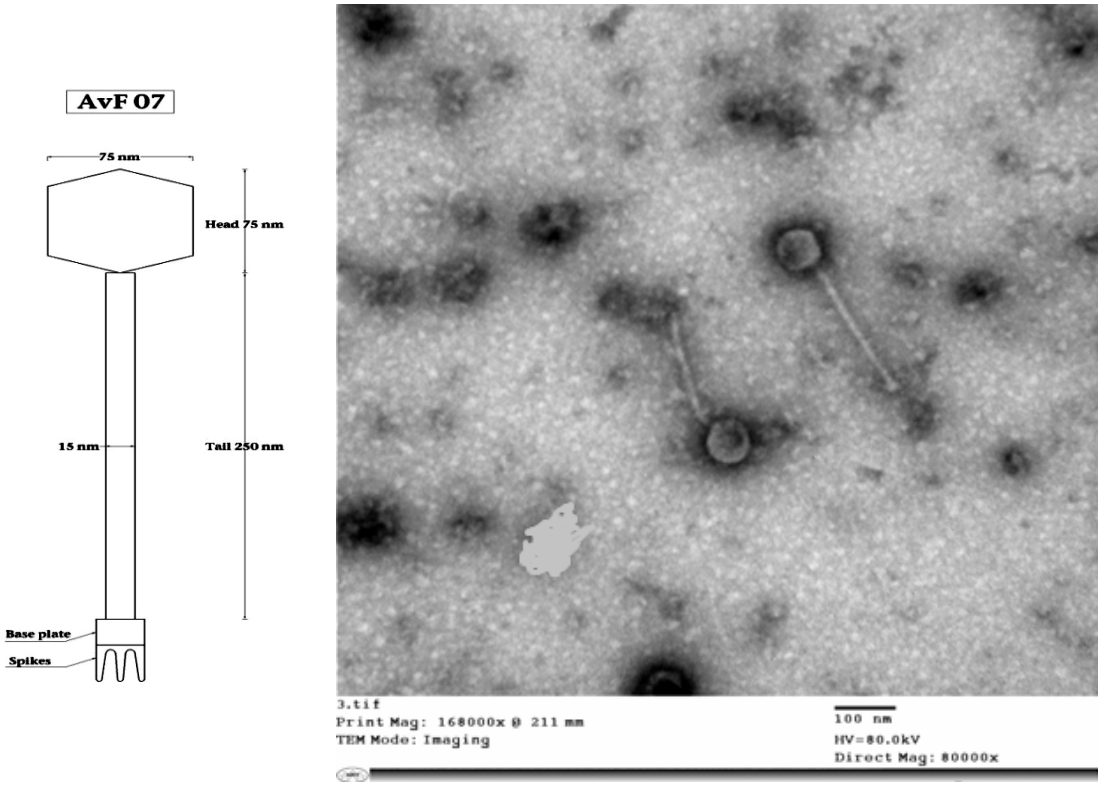


Fig. 3. Schematic diagram and electron micrograph of negatively stained phage AvF07. The bar represents 100nm

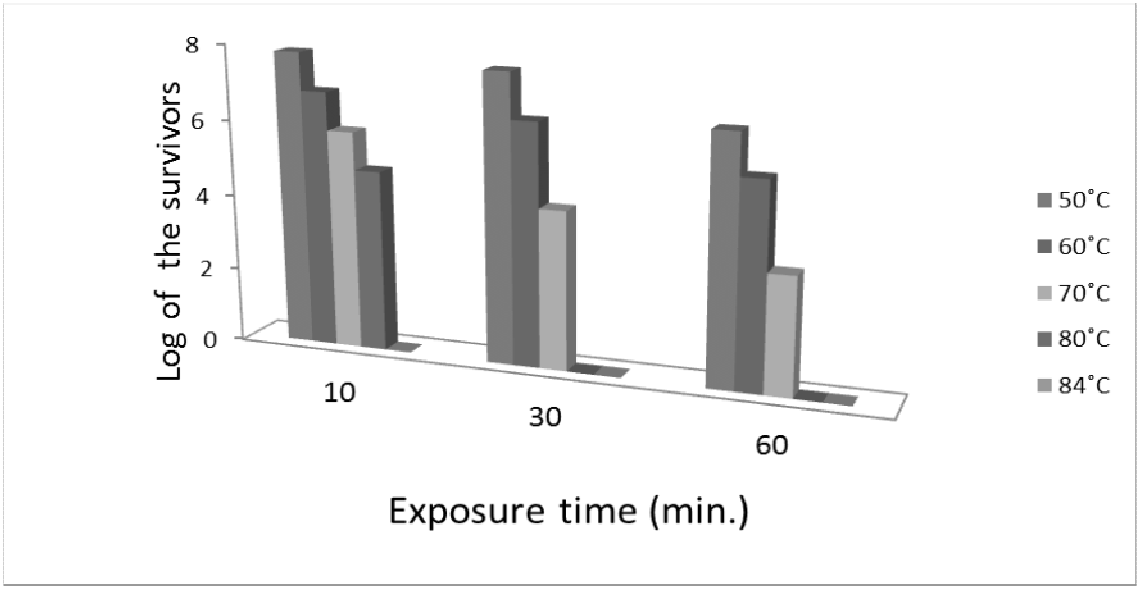


Fig. 4. Effect of temperature degrees on the viability of phage (AvF07)

However, Phage AvF07 was sensitive to storage at 4°C compared to storage at -20°C since it lost its activity between 2-4 months at 4°C, the reduction being 18.7 and 56.2% when the phage was stored for 2 and 4 months, respectively. These results were comparable with those obtained by **Chow** and **Rouf (1983)** they found that on day 60th of storage at 4°C, phages Aeh1 and Aeh2 were decreased to 60 and 65% of their original infectivities , respectively. While **Jepson** and **March (2004)** reported that good phage stability was found when phage λ was stored at 4°C for over 6 months. Concerning phage AvF07, 4°C is considered optimum for short storage (no longer than 4 months) phage storage. In this connection **Olson *et al*. (2004) and Rai *et al*. (2018)** stated that an optimum temperature for short time (no longer than 40 days) was at 4°C for phage MS2 storage. Phage (AvF07) was resistant to storage at -20°C since its viability retained after 6 months but lost it's infectivity after more than 6 months (100% reduction).

Effect of pH on phage AvF07

From Fig. 5 it can be noticed that phage AvF07 showed variable levels in sensitivity to different pH values being stable in acidic side reduction in survival was only 61.2% at pH 2 and relatively stable within a pH range of 4-10 since the reductions at the two extreme points were less than 50%. Reduction in the survival of phage reached to log of 3.8 and 5.8 log at pH 4 and 11, respectively played greater. These results are in agreement with those obtained by **Chow** and **Rouf (1983)** they found that the range of pH which for phage *A. hydrophila* Aeh1 was stable at 5-10 while this range was 5-9 for phage Aeh2. Both phages lost their activities totally at pH 3 or 11 for an hour at 22°C. Also our results are in harmony with those obtained by **Han *et al*. (2014), Taj *et al*. (2014)** as well as **Phumkhachorn and Rattanachaikunsopon (2018)** who stated that phage φPA-HF17of *Pseudomonas aeruginosa* and phage T4 of *E. coli* were stable over a wide pH range (5-10) and (4-10), respectively. Generally, phage (AvF07) in this investigation was stable in a broad range of pH (2-11), more sensitive to the alkaline condition and less sensitive to acid side.

In vivo Effects of Phage, Antibiotics or Nanoparticles on *Aeromonas veronii* AFs_2 Strain

Aeromonas veronii AFs_2 was a primary or secondary cause of skin darkness, scales detachment, blindness and large irregular hemorrhages on the body surface, fin necrosis, exophthalmia, hemorrhage septicemia, and eye cataract/trachoma in fish **(Shayo *et al*., 2012)**.

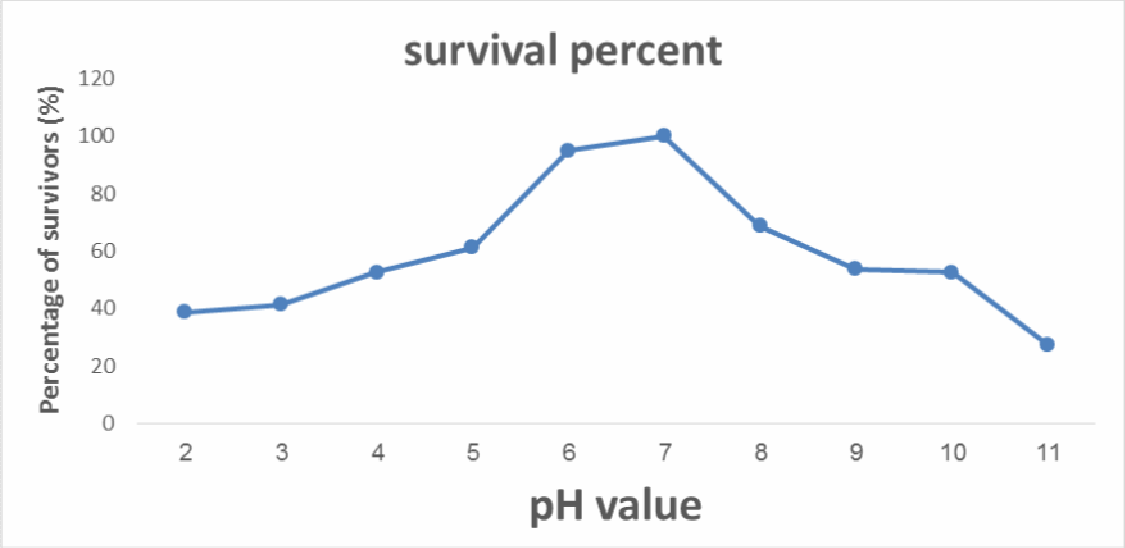


Fig. 5. Effect of pH value on the survival of phage AvF07

Aeromonas veronii AFs_2 was highly resistant to 19 antibiotics while susceptible to phage AvF07 therefore this species was chosen for studying its sensitivity to silver nanoparticles as well as chitosan nanoparticles turbidometrically and consequently in application experiment.

Without major effects on the structure of natural bacterial communities of aquaculture waters, phage therapy may represent a viable alternative to antibiotics to inactivate fish pathogenic bacteria **(Pereira *et al.*, 2011)**. Since phage AvF07 presented stable feature in storage conditions at different temperatures as well as in various pH and has a broad host range of *Aeromonas* spp. An evaluation of the effect of silver nanoparticles "AgNPs" (20 µg/fish), an eco-friendly application of chitosan nanoparticles (0.2 µg) *in vivo* as antibacterial agent against *Aeromonas veronii* AFs_2 in fish aquarium, chloramphenicol (30 µg/fish) and phage AvF07 (1.3×10^6 PFU/fish) on the mortality of Nile tilapia fish as well as fish previously intrapretenualy injected with either $2.3x10^6$ CFU/fish was done (Table 6). Also, many *in vivo* studies demonstrated conflicting results against use of AgNPs in fish **(Lee *et al.*, 2012; Scown *et al.*, 2010; Márquez *et al.*, 2018)**.

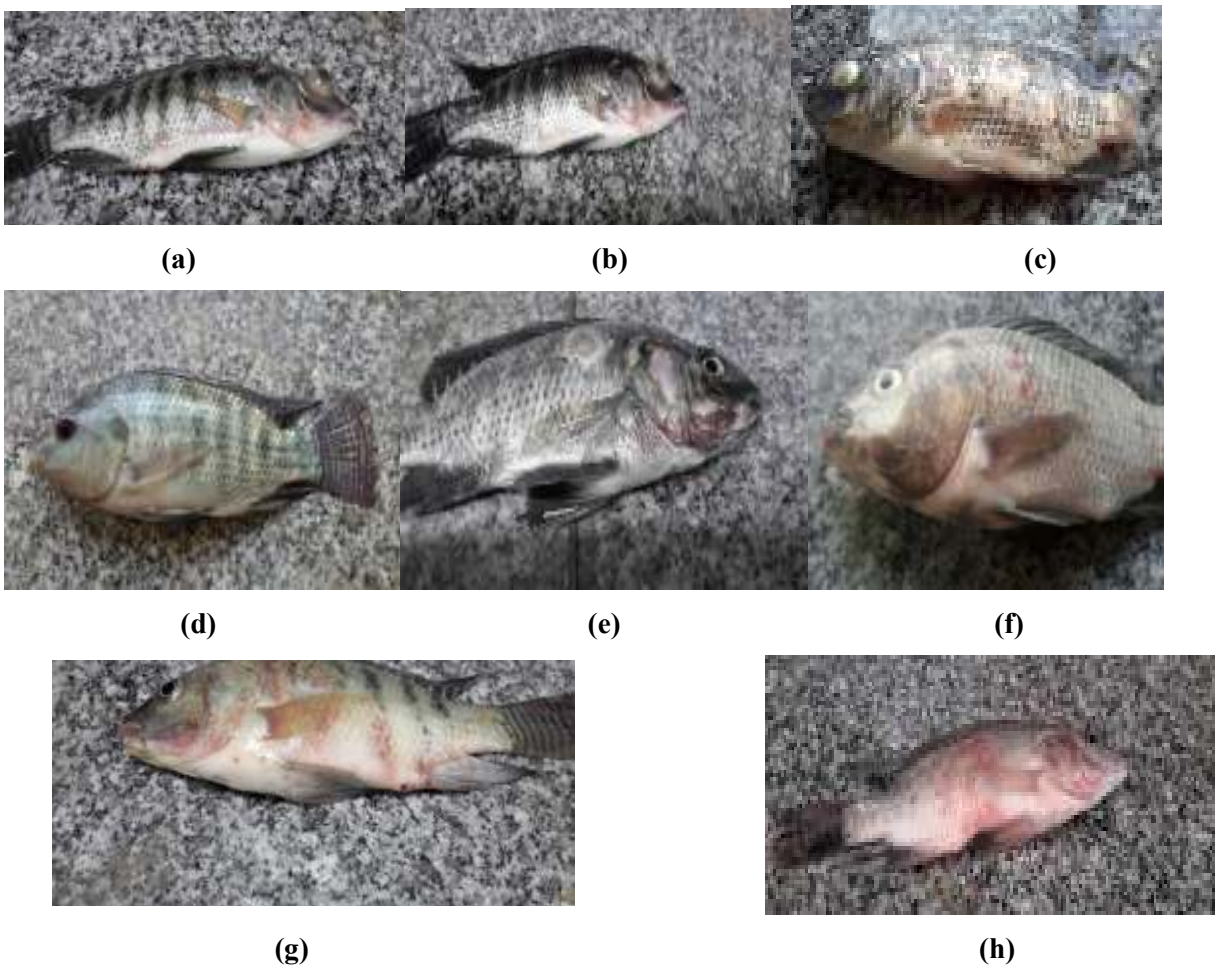
The effects of silver nanoparticles (AgNPs), chitosan nanoparticles, chloramphenicol and phage AvF07 on the virulence of *Aeromonas veronii* AFs_2 in fish are shown in Table 6. When phage (AvF07) was used in combination with *A. veronii* AFs_2 using intraperitoneal injection, no mortality was shown until the end of experiment (14 days). On the contrary, when chloramphenicol was injected in stead of phage it was shown that 43.8% and 50% mortalities were detected after 2 and 3 days, respectively. On the other hand, no mortality as well as no clinical abnormality on fish preinjected with either only silver nanoparticles or phage each or incombination with *Aeromonas veronii* AFs_2 ($2.3x10^6$ CFU/fish) were shown. However, fish injected with bacterial suspension alone showed firstly lethargic movements and secondly abnormalities on fish with the low dose of bacteria Fig. 6 followed by a mortality of the fish after 4 days, when *A. veronii* AFs_2 was injected with the dose of $2.3x10^6$ CFU/fish. Therefore, phage therapy could be used as a sustainable biological control for the reduction of *Aeromonas* spp. in aquaculture. When phage (AvF07) was used in combination with *A. veronii* AFs_2 using intraperitoneal injection (Table 6).

In conclusion, bacterial resistance to antibiotics is a growing threat in our world. Multidrug-resistant bacteria have opened a second window for phage therapy which can then serve as a stand-alone therapy for infections that are fully resistant. It will be also then able to serve as a co-therapeutic agent for infections that are still susceptible to antibiotics, by helping to prevent the emergence of bacterial mutants against either

Table 6. *In vivo* effect of antibiotics, nanoparticles or phage AvF07 on the pathogenicity of *Aeromonas veronii* AFs_2 in fish

Treatment*	Fish No.	Number of death / day													
		1	Av. mo.(%)	2	Av. mo.(%)	3	Av. mo.(%)	4	Av. mo.(%)	5	Av. mo.(%)	6	Av. mo.(%)	7	Av. mo.(%)
Control without injection	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Water	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chloramphenicol 30µg/fish	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AgNPS 20µg/fish	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chitosane 200ng/fish	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phage only AvF07 $1.3x10^6$ pfu/fish	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A.veronii* AFs_2 *	16	0	0	5	31.3	9	56.3	2	12.5	-	0	-	0	-	-
***A.veronii* AFs_2 +Chloramphenicol**	16	0	0	0	0	0	0	7	43.8	8	50	1	6.3	-	-
***A.veronii* AFs_2 + AgNPs**	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.veronii* AFs_2 + Chitosan**	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.veronii* AFs_2 + Phage AvF07**	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* All treatments were accomplished using intra-peritoneal injection. (0) : no mortality. , (-): 100% mortality. , Av.mo.(%): Average of mortality, No.: number. (**): *A.veronii* AFs_2 with a concentration of $2.3x10^6$ cfu/fish.



(a) (b) (c)

(d) (e) (f)

(g) (h)

Fig. 6. Nile-tilapia fish experimentally infected with *Aeromonas veronii* AFs_2 showing (a,b) skin darkness, exophthalmia, eye cataract/trachoma, scales detachment, (c) blindness, (d) healthy fish, (e) hemorrhagic septicemia, and (e,f,g) large irregular hemorrhages on the body surface and (h) fin necrosis

agent. Though many believe that phages will not replace antibiotics right away or may be ever, there is definite potential for their use in conjunction with antibiotics. Still, with the rise of *Aeromonas* antibiotic resistance, bacteriophages may be able to offer a line of defense in situations for which antibiotics are not available, or are not effective. Also, in this scenario, the antimicrobial efficacy of silver nanoparticles or chitosan nanoparticles against the fish pathogen *Aeromonas veronii* AFs_2 generates hope for its possible application as a disinfectant or antimicrobial agent for better fish health management. However, further *in vivo* experiments have to be carried out for safety assessment of silver nanoparticles and/or chitosan nanoparticles as well as phagetherapy before any large scale application.

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عزل بكتيريوفاج الإيروموناس (AvF07) من السمك واستخدامه للمقاومة البيولوجية لبكتيريا *A. veronii* AFs_2 المحلية ذات المقاومة المتعددة للمضادات الحيوية

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تم تعريف بعض من الإيروموناس المعزولة من السمك البلطي والأحواض السمكية ومياه نهر النيل هذا بالإضافة إلى عزل البكتيريوفاج الخاص بها، هذا بجانب تقييم التأثير المضاد لكل من جزيئات النانو والفاج على الـ *A. veronii* AFs_2 الممرض، تم التمييز بين أنواع الـ *Aeromonas* من خلال ٢٥ اختبارا بيولوجيا مختلفا وتم التحقق منه بتقدير تتابعات جين 16S rRNA وكانت (*A. caviae* AFg, *A. encheleia* AWz, *A. molluscorum* AFm, *A. salmonicida* AWh, *A. veronii* AFs_2, *A. veronii bv. veronii* AFi). كما أظهر اختبار المقاومة للمضادات الحيوية أن كل سلالات الإيروموناس كانت مقاومة لمضادات بيتالاكتام (amoxicillin/clavulanic acid)، ومن جهة أخرى تباينت مقاومة الإيروموناس للمضادات الحيوية الأخرى، حيث كانت كل سلالات الإيروموناس المدروسة مقاومة للأمبيسيلين، السيفاليكسين، السيفرادين، الأموكسيسيلين/كلافيولانيك أسيد، الريفامبين وكذلك السيفالوثين، وكان هناك تباين في حساسية ٦ سلالات الإيروموناس المختبرة تجاه ٧ تركيزات من جزيئات الشيتوزان النانونية، كما لوحظ نشاط كبير لجزيئات الفضة النانونية (بحجم ٢٠ نانوميتر) مضاد للميكروبات باستخدام طريقة الانتشار في جوَر الآجار عند تركيز ٢٠، ٢٤، ٣٢، ٤٠ ميكروجرام لكل مللي، وحددت نقطة الحرارة المفقدة لنشاط الفاج (AvF07) بـ ٨٤ درجة مئوية بينما كان الفاج حساسا للتخزين عند ٤ درجة مئوية مقارنة بالتخزين عند -٢٠ درجة مئوية، وعند إستخدام فاج AvF07 مع بكتيريا *A. veronii* AFs_2 باستخدام الحَقن تحت الغشاء البريتوني للسمك ، لم تسجَل حالة نفوق حتى نهاية التجربة (١٤ يوم)، وكانت نسبة النفوق المتحصل عليها ٤٣,٨% أو ٥٠% بعد ٢ أو ٣ أيام على التوالي عند استخدام الكلورامفينيكول بدلا من الفاج.

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