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Isolation and Characterization of Aeromonas hydrophila lytic Bacteriophages as a safe antibiotic alternative bio-control agent

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Abstract. This study aimed to develop a safe bio-control approach for therapy and decontamination of *Aeromonas hydrophila.* The study focused on determination of the drug susceptibility of *Aeromonas* isolates from diseased tilapia; and establishment of a stock of partially characterized *Aeromonas hydrophila* bacteriophages. Eighteen (18) *Aeromonas* spp isolates were tested for antimicrobial resistance against 10 antibiotics. The susceptibility of the isolates to various antibiotics ranged from 0 to 100%. For bacteriophages, three lytic phages that had the highest host range (78.6% to 92.8%, n=28) were selected. Further characterization revealed a burst period of 20 ± 5 to 40 ± 5 minutes, a latent period that varied from 10 ± 5 to 40 ± 5 minutes; and burst size of 98–171 virions per infected cell. There were no significant changes in phage titers on exposure at 40°C and 50°C for 60 minutes (P = 0.05). However, a gradual decrease in the titres was observed at 60°C while a fast decline was noted at 70°C-90°C. All phages demonstrated at least some activity in the pH range of 1-13. In water stability testing, the phages survived up to 22 days of the experiment, with a sharp decrease of 1 log PFU/mL to have been encountered within the first day. The findings of this study suggest phages as bio-control agents against Aeromonas infection in fish. However, further genomic characterization is required.

Keywords: Bacteriophage, Antibiotic resistance, aeromonasis, Aquaculture, Bio-control.

Introduction

In response to the declining capture fisheries worldwide, aquaculture is increasingly introduced to fill up the gap; and the Uganda government supports this sector for nutrition and economic security (Mbowa *et al.*, 2017; Wamala *et al*, 2018). Due to commercial and production reasons, farmed fish are held in much more crowded conditions (Annabi et al., 2015) which might enhance the spread of pathogenic bacteria and cause serious outbreaks of diseases. Bacterial infections contribute to the low survival rate of the affected fish and these cause significant challenges to fish farmers (Pekala-Safinska, 2018). Given that these pathogens including

Aeromonas hydrophila can survive well in the aquatic environment independently of their hosts, they became major impediment to aquaculture (Semwal et al., 2023; Zhou et al., 2019). Disease management involves both prophylactic and therapeutic use of antibiotic-supplemented feeds and mixing drugs in pond water (Au-Yeung et al., 2022; Griboff et al., 2020). Unfortunately, the indiscriminate use of antimicrobial agents has led to the development of resistant strains of bacterial pathogens (Bunnajirakul *et al*., 2015).

Infections caused by antibiotic-resistant *A. hydrophila* are difficult to treat, and the fact that there are only a few new antimicrobial compounds in the drug development pipeline complicates matters even more (Eid et al., 2022; Yin et al., 2020). Similarly, antibiotics may inhibit growth and compromise the defines mechanisms of fish larvae (Bunnajirakul *et al*., 2015). Studies have reported the excretion of antibiotics administered to fish via faeces and through gills (Chen et al., 2020; Rigos et al., 2004). This situation results in antibiotic contamination of the environment, which is one of the drivers of drug resistance development. As a result, alternative management strategies for *A. hydrophila* infected fish are required.

As antimicrobial resistance to commonly used antibiotics become a challenge, bio-control agents such as lytic bacteriophages can be a potential option. They can be applied to prevent and treat multidrug resistant bacterial infection in farmed fish. Phages are relatively easy to isolate compared to discovering new drugs. The need for alternative strategies for minimizing drug use and management of antimicrobial resistance has stimulated a lot of research into bacteriophages (Kalatzis et al., 2016). The use of phage therapy has several advantages: bactericidal agents, auto "dosing", low inherent toxicity, minimal disruption of normal flora (specificity), the narrower potential for inducing resistance, lack of cross-resistance with antibiotics, rapid discovery, formulation, and application versatility and biofilm clearance (Liu et al., 2022).

Aquaculture, promoted to address the decline in capture fisheries, exposes farmed fish to stressful conditions (Bartley, 2022). This stress predisposes them to disease outbreaks, which are mainly managed by maintaining proper hygiene and administering antibiotics (Ahmad et al., 2021; Bartley, 2022). However, antibiotic use has been reported as the major driver for the development of antimicrobial resistance (Mog et al., 2020). *A. hydrophila* is one of the common bacterial pathogens of freshwater fish, with a prevalence of 43.8% reported along the Lake Victoria Basin (Wamala et al., 2018). The bacteria exhibit drug resistance frequencies of 23.2% and 50% to Cefotaxime and Oxacillin, respectively, and all strains show resistance to Erythromycin, Ampicillin, and Penicillin (Wamala et al., 2018). The use of antibiotics in animal production systems is heavily regulated in developed countries (EFSA et al., 2021). In developing countries, there is a growing awareness, and governments are working towards reducing the use of antibiotics. Therefore, there is a need to establish alternative strategies for controlling fish bacterial pathogens, specifically *A. hydrophila*. Bacteriophages are currently considered promising candidates worldwide (Pirnay *et al*., 2015).

This study aimed at evaluating *Aeromonas hydrophila* specific bacteriophages obtained from pond sediment, water, and fish gastrointestinal tract collected from different agroecological zones of Uganda, to contribute to the global antimicrobial resistance action plan.

Methodology

Cryopreserved *Aeromonas* ssp. stock bacterial isolates were resuscitated in single-strength Trypticase Soy Broth (TSB) and incubated at 28°C overnight. The bacteria were originally isolated from diseased Tilapia and kept at the Veterinary Microbiology Laboratory at the College of Veterinary medicine Animal Resources and Biosecurity, Makerere University

(COVAB). From the overnight TSB culture, sub-cultures were made on Trypticase Soy Agar (TSA) to obtain pure cultures. All the media used were obtained from Condalab, Madrid, Spain; except for MacConkey agar from OXOID, United Kingdom and Bile salts Irgasan brilliant green (BSIBG) agar from Himedia, India. The isolates were characterized by colony morphology, lactose fermentation on MacConkey agar (OXOID, UK), hemolytic activity on Blood agar (BA) and growth on BSIBG. Tentative species identification was achieved using selected biochemical tests as described by (Piotrowska & Popowska, 2014). *A. hydrophila* strain F7 is available in the public Genbank database via accession number CP138197 which was characterized and identified during previous research.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using the disk diffusion method following the recommendation from the Clinical and Laboratory Standards Institute (CLSI, 2020). Ten antibiotics, including Tetracycline (25μg), Chloramphenicol (5μg), Nalidixic acid (30μg), Ampicillin (10μg), Streptomycin (10μg), Ciprofloxacin (5μg), Penicillin G (10μg), Trimethoprim/sulfamethoxazole (25μg), Nitrofurantoin (300μg), and Gentamicin (30μg); from Bioanalyse®, were tested for their bacterial growth-inhibitory activity on Mueller-Hinton agar. Sizes of growth-inhibition zones were recorded and interpreted as susceptible (S), intermediate (I), and resistant (R) following the CLSI Vet04 (CLSI, 2020).

Sample sources for bacteriophage isolation

Pond sediments (17) and fish gastrointestinal tract (30) samples were collected from fish ponds from selected farms in the Central, Eastern, Northern and Western regions of Uganda. The samples were transported on ice in a cool box to the Veterinary Microbiology Research Laboratory; where they were processed and added to the SM buffer (0.05M Tris, 0.1M NaCl, 0.008M $MgSO_4$, 0.01% w/v gelatin, pH 7.5) and kept at 4°C in a refrigerator. The sample-SM suspension (80:20 v/v) was vortexed, transferred to a 15ml falcon tube then centrifuged at 6000 rpm (Hermle Z32K, Germany) to obtain the supernatant. The supernatant was transferred to a sterile falcon tube and the sediment was discarded after autoclaving. The supernatant analysed further to obtain the bacteriophages. *Samples were pooled together prior to phage isolation

Phage isolation, purification, amplification, and characterization

The bacteriophages were isolated using the enrichment method as described by Kalatzis *et al.*, (2016) and Haq, Chaudhry, Andleeb, & Qadri, (2012). Briefly, the sample-SM supernatant was filtered through 0.45μm filters (ADVANTEC®, USA) and 10ml of the filtrate was added into the flask containing an equal volume of double strength Tryptose Soy Broth (TSB) supplemented with 2 mls of 0.05M Calcium Chloride, and then 100 μl of the overnight *Aeromonas hydrophila* F7 culture was added. The flask was incubated at 28°C for up to 24h with shaking at 250 rpm in the incubator shaker (New Brunswick™ Innova® 40, Germany). After 24h of incubation, 15ml of the sample was collected, centrifuged at 15,000×g for 10 minutes, and filtered through 0.45μm and the filtrate was stored at 4°C until required.

Spot assay method

The spot assay method was used for the detection of bacteriophages in a suspension, host range and quantitation of the virus particles. The bacterial lawns were prepared by mixing the semi-solid media of TSB broth containing 0.7% of the bacteriological agar, with 100μl of overnight *A. hydrophila* culture. The mixture was then poured evenly on a TSA plate to make a double

layer as described by (Santos et al., 2009); with slight modifications. Using sterile pipette tips, 10µl of the filtered samples were spotted onto a section of the agar plate; allowed to dry, and then incubated at 30°C overnight. The presence of bacteriophages in the preparation was indicated by the appearance of clear areas or plaques due to growth-inhibition activity.

Plaque assay

The plaques assay was employed for purification and quantitation of the bacteriophages in a given suspension. The assay involved the preparation of an agar overlay comprised of TSB with 0.7% agar in a test tube and the bottom comprised on TSA in a Petri dish. Agar overlay or plaque assay as described by Khan Mirzaei and Nilsson (2015) was used.

Briefly, the top layer, which comprised of preparation of semi-solid TSB by adding 0.7% bacteriological agar. The media was dispensed in 4 mls volumes and maintained in a molten form at 50°C in a water bath (memmert, Germany). The bottom layer consisted on TSA dispensed in Petri dishes. The phage suspension, 100µL was mixed with 100µL of an overnight bacterial culture and then added to the 4mls of agar overlay. After proper mixing by gentle inversion, the contents were poured over the TSA bottom layer, and the plate was gently moved to allow even spreading of the agar overlay. The plate was kept at room temperature to allow solidification of the agar, after which it was placed in an incubator (memmert, Germany) at 28°C for overnight.

Phage purification

For phage purification, the plates were examined for distinct plaques were morphological characterisation by shape, size, and opacity. The representative plaques with different morphologies were independently harvested using a sterile pipette tip. The harvest was done by simply touching a plaque with the tip and washing the adhering phages into a small volume of SM buffer in a microfuge tube. The process was repeated thrice to ensure the purity of the phage preparations. The plaques were described as big or small, with halo or without, transparent or translucent.

From a plate with similar plaques, phages were harvested by flooding with 1ml of SM buffer, left for 30 min, after which it was collected into a microfuge tube and kept at 4°C until further phage characterization, but not beyond 7 days.

Phage Quantitation

The pure phage preparation was subjected to 10-fold serial dilution using cold SM buffer as the diluent. To each Eppendorf tube, properly labelled with the respective dilution factor, 100µl of the overnight culture was added; and similarly, one TSA plate had the bottom marked out into sections, labelled with dilution factors to correspond with those on the Eppendorf tubes from 10^{-1} to 10^{-10}. Ten tubes of melted overlay agar were prepared, then 0.3 ml of a broth overnight culture of *A. hydrophila* was dispensed in each tube using a sterile pipette tip, each tube was mixed well and put in the water bath to maintain the agar in a liquid form.

Sterile tubes each containing 900µl (0.05M Tris, 0.1M NaCl, 0.008M $MgSO_4$, 0.01% w/v gelatin, pH 7.5) were prepared and labelled from 10^{-1} through 10^{-10}. Ten TSA plates were labelled with concentration corresponding the SM tubes. Using sterile pipettes, a 10-fold serial dilution was made by transferring 100µl of the bacteriophage suspension to the 1st tube (labelled 10^{-1}), mixed well by rolling between the palms, and then a fresh a sterile pipette was used to transfer 0.5 ml of the solution to the next tube. The process was repeated until the 10th tube.

From each phage dilution, the agar overlay method was performed. After incubation, each plate was examined for the presence and number of plaques. Plates with plaques between 30 – 300 were selected for phage quantitation (Kropinski et al., 2009). The phage concentration in the original pure preparation was calculated using PFU calculator tool (Lwesya, 2019).

Phage characterisation

Characterisation of bacteriophage isolates involved comparison of phage yield at selected multiplicity of infection (MOI), host range, one step growth curve and thermo stability. All experiments were done in triplicates

Host range

The host range was established by the spot assay, as described above. Phage activity was determined using 23 *Aeromonas* spp including *Aeromonas hydrophila* (14), *Aeromonas caviae* (6), and *Aeromonas veronii (3).*

One-Step Growth Curve

One-step growth curve analysis for comparing selected phage isolates by latent period, burst time, and burst size was conducted according to Verma *et al.* (2009) with some modifications (Verma et al., 2009). Briefly, 10 ml phage suspension (10^7 PFU/ml) was added to a 20-ml overnight culture (10^{10}CFU/ml) of the host bacterium and the mixture was incubated at 30 ℃ for min. After centrifugation at 35,000× g for 30 min, the supernatant was carefully removed and the pellet was suspended in 20 mL nutrient broth. The mixture was incubated at 30℃ for 110 min, and samples were collected at 10-min intervals. The aliquots were serially diluted, and phage titers were determined by the overlay agar method.

The latent period was the period that was obtained as the interval from time=0 to just before the burst period commences. Burst time was the time at which maximum phage titres were observed (plateau phase) while burst size was obtained by the formula:

$$\text{Burst size} = \frac{Bacteriophage\ concentration\ at\ plateu\ phase\ (\frac{PFU}{Ml})}{Bacteriophage\ concentration\ at\ the\ latent\ period\ (\frac{PFU}{Ml})}$$

Comparison of phage lytic activity by Multiplicity of Infection

Multiplicity of infection was performed as described by Abedon (2016) with slightly modification in incubation time (Abedon, 2016). The host isolates were subjected to MOI of 10, 1, 0.1 and 0.01 and then incubated for 48 hours. The bacteriophage population were quantified at 24 hours and at 48 hours of incubation. The experiment was done in duplicates, and results recorded as average phage concentration and standard deviation for each MOI at the two time points.

Evaluation of the Effect of temperature of phage stability

Phage stability studies were performed according to protocols described by Mirzaei & Nilsson, (2015); Topka *et al*., (2019). The stability of isolated phages was tested against a wide temperature range (40℃, 50℃, 60℃, 70℃, 80℃, and 90℃) in triplicates described by (Harwood *et al*., 2013). The working stock (10^7 PFU/ml) of each phage (100 μL) was suspended

in 900 μL PBS and incubated at 40℃, 50℃, 60℃, 70℃, 80℃, and 90℃ for 60 min in a water bath. The phage titer at each temperature was determined every 10 min during the 60-minute incubation using the spot assay after serial dilution of the phage preparation.

For cold storage stability, a 10 ml stock solution of each isolated phage was stored at 4℃, and −20℃, and sampled at 0, 1, 7, 14, 45, and 90 days. Survived phage populations were quantified.

Data and statistical analyses

The results obtained from the laboratory experimentation was entered into Excel®, sorted and cleaned, thereafter, exported to SPSS for analysis using ANOVA to determine if there was any variance on phage concentration subjected to a different temperature and data was presented as the average and standard deviation for thermo stability experiments.

Research approval

Research approval was obtained from the Institutional Review Board School of Biosecurity, Biotechnical and Laboratory Sciences (SBLS) of College of Veterinary Science, Animal Resources and Biosecurity, reference number SBLS/HDRC/19/010.

Results

Antimicrobial susceptibility of *Aeromonas* spp

Table 1 presents the results of the antimicrobial susceptibility tests for the selected *Aeromonas* spp strains. Out of the 18 *Aeromonas* spp isolates tested against the 10 antibiotics, 100% sensitivity was encountered for Ciprofloxacin (Cip) and Gentamycin (CN). For the rest of the antibiotics, the proportions of sensitive isolates were as follows: Chloramphenicol (C) 61.1%; Penicillin (P) 33.3%; Tetracycline (TE) 55.5%; Nalidixic (NA) 50.0%; Nitrofurantoin (F) 38.9%; Streptomycin(S) 50.0%; and Trimethoprim/ Sulphamethoxazole (SXT) 44.4%. All the isolates were resistant to Ampicillin (Amp), while 66.7% exhibited multi-drug resistance. The phage isolation bacterial strain, *A. hydrophila* F7, was sensitive to 50% of the drugs.

Isolation of Bacteriophages

From the 47 samples (17 from pond sediment and 30 from fish GIT) 19 crude phage preparations were obtained. On purification and basing on plaque morphology, three phages, specific to *A. hydrophila* strain F7, were selected and designated laboratory identifications as Ahyd1, Ahyd2, and Ahyd3.

Host Range Determination of Bacteriophages

Host range determination considered the number of bacterial strains lysed and the intensity of clearance. The intensity of host lysis encountered is presented in Fig. 2.1. The host range of the isolated phages was determined against 23 *Aeromonas* spp isolates.

Table 2.1: The antimicrobial resistance pattern of *Aeromonas* spp

	Antimicrobial agent									
Isolate ID	*C*	*CIP*	*P*	*TE*	*AMP*	*NA*	*F*	*S*	*CN*	*SXT*
F7*	R	S	R	S	R	S	S	R	S	R
AERO S2	S	S	R	S	R	I	S	R	S	S
Aeromonas (MUKONO)	R	S	R	R	R	R	R	R	S	R
Aeromonas spp 1	R	S	R	I	R	R	R	R	S	R
Aeromonas spp 2	R	S	R	R	R	R	R	R	S	R
Aeromonas spp 3	I	S	R	I	R	I	R	S	S	I
Aeromonas spp 4	S	S	S	S	S	S	S	S	S	S
Ah2	S	S	S	S	S	S	I	S	S	S
Ah2B	R	S	R	R	R	R	R	R	S	R
Ah3 *A. hydrophila*	S	S	S	S	S	S	S	S	S	S
Av1(*Aeromonas veronii*)	S	S	S	S	S	S	S	R	S	S
Av2(*Aeromonas veronii*)	R	S	R	I	R	R	R	S	S	R
K tank	S	S	S	S	S	R	R	S	S	R
K tank 2	S	S	R	S	R	S	S	S	S	I
KADRC	S	S	S	S	S	S	S	S	S	S
KADRC2	S	S	R	R	S	S	R	I	S	R
S Aero	S	S	R	S	I	R	R	I	S	S
S Aero2	S	S	R	I	R	S	R	S	S	S

Key: S – Sensitive, I – Intermediate, R – Resistant, C – Chloramphenicol, P – Penicillin, TE – Tetracycline, Amp – Ampicillin, NA – Nalidixic, F – Nitrofurantoin, S - Streptomycin, SXT - Sulphamethoxazole, Cip - Ciprofloxacin, CN - Gentamycin, * - Phage isolation host

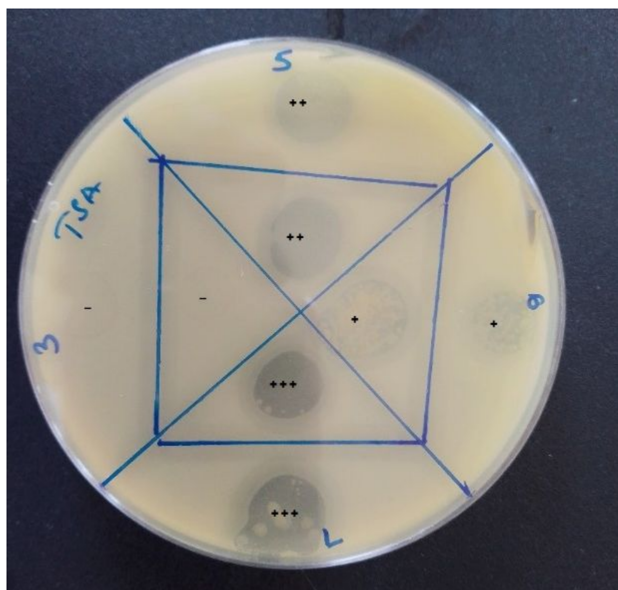

Figure 2.1: Phage sensitivity of Aeromonas spp to selected phages

Note the different clearance spots that were used for categorization of phage sensitivity during host range experiments. Complete clearing (+++), clearing throughout but with a faintly hazy background (++), turbid clearance (+), and no clearance (-).

The activity of 19 phage isolates against the 23 *Aeromonas* spp strains, ranged from 0 – 100%, with three isolates, coded Ahyd1, Ahyd2, and Ahyd3 lysing 100%, 86.3%, and 95.5% of the bacteria. Table 2.2 presents the bacteria sensitivity to the three selected bacteriophages. Phage Ahyd1 formed complete clear (+++), clearing throughout but with a faintly hazy background (++) and turbid clearance (+) plaques on 59%, 22.7%, and 13.6% of the *Aeromonas* spp isolates, respectively. Phage Ahyd2 formed clear (+++), mild clearance (++), and turbid clearance on 46.4%; 22.7% and 22.7% *of the Aeromonas* spp isolates, respectively; while no activity was observed on three (13.6%) bacterial isolates. Phage Ahyd3 formed clear (+++), mild clearance (++), and turbid clearance on 31.81%; 40.9%; and 18.2% of the isolates, respectively; but only one strain (4.5%) was not sensitive. By combined phage activity, the host range was 100%. Bacterial isolate AH2b was sensitive to only one bacteriophage, Ahyd1, which produced turbid clearance. However, other isolates were sensitive to two or more phages with at least one exhibiting clear plaques.

Table 2.2. Phage activity on different *Aeromonas* sp. and other related bacterial isolates

Bacterial isolate		Phage Isolate Identification		
Lab ID	*Tentative spp identity*	*Ahyd1*	*Ahyd2*	*Ahyd3*
Aero p	*Aeromonas hyrophila*	++	+	+++
Aero p S2	*Aeromonas caviae*	+++	++	+++
Aero p2	*Aeromonas hyrophila*	+	+	++
AERO S1	*Aeromonas hyrophila*	+++	+++	++
AERO S2	*Aeromonas hyrophila*	+++	+++	+++
Aeromonas (MKN)	*Aeromonas hyrophila*	+++	+++	++
Aeromonas spp 1	*Aeromonas caviae*	+++	++	++
Aeromonas spp 2	*Aeromonas hyrophila*	+++	+	+
Aeromonas spp 3	*Aeromonas hyrophila*	+++	+++	+
Aeromonas spp 4	*Aeromonas veronii*	++	+++	+++
Ah2	*Aeromonas caviae*	+++	+	+
Ah2B	*Aeromonas hyrophila*	+	-	-
Ah3	*Aeromonas hyrophila*	+++	+++	++
Av1	*Aeromonas veronii*	+++	++	++
Av2	*Aeromonas veronii*	+++	+++	++
K tank	*Aeromonas caviae*	+	+++	++
K tank 2	*Aeromonas caviae*	++	+	+++
KADRC	*Aeromonas hyrophila*	++	-	+++
KADRC2	*Aeromonas caviae*	+++	-	+++
S Aero	*Aeromonas hyrophila*	++	++	++
S Aero2	*Aeromonas hyrophila*	+++	++	+
F7*	*Aeromonas hyrophila*	+++	++	++
HOST RANGE (n =22)		89.3%	78.6%	92.8%

Key: Complete clearing (+++), clearing throughout but with a faintly hazy background (++), turbid clear substantial turbidity throughout the cleared zone (+) and no clearance (-), * Phage isolation host

Latent time and burst sizes

The one-step growth curves were constructed by plotting a graph of phage titers against time and were used to compare the latency period, burst time, and burst sizes of the different phage isolates. As presented in Fig. 2.2, the latent time of Ahyd1, Ahyd2, and Ahyd3 was estimated as

40, 30, and 10 minutes respectively; while the burst size of these phages was 171 PFU, 98 PFU, and 150 PFU per infected cell, respectively.

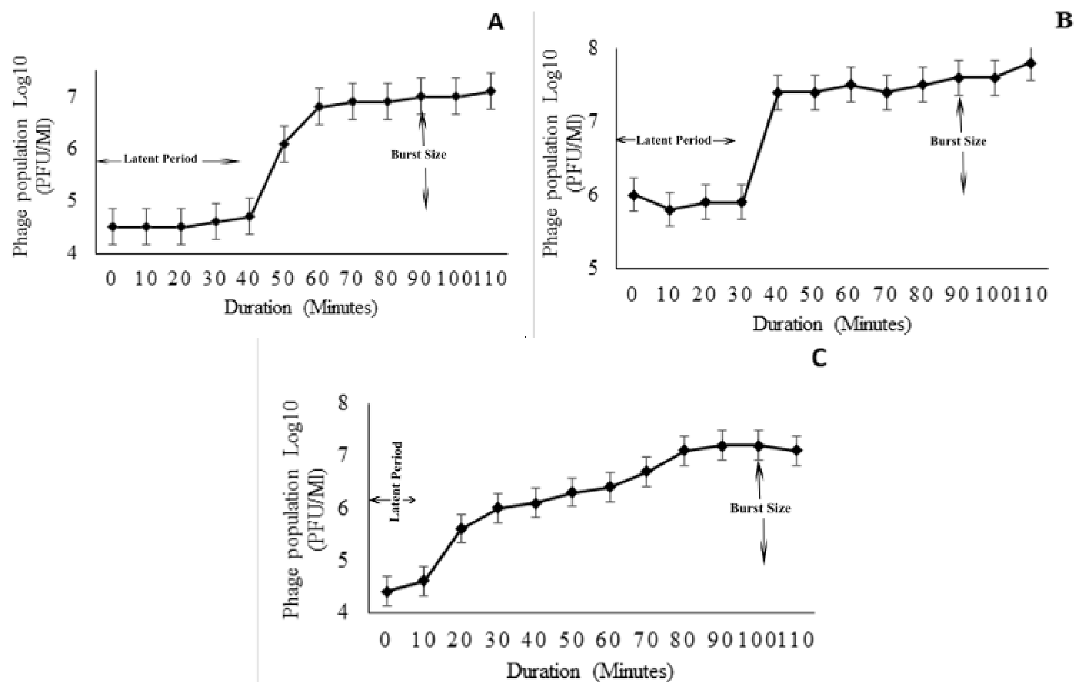

Figure 2.2: One-step growth curve for phage (A) Phage Ahyd1 (B) Phage Ahyd2 and (C) Ahyd3

Comparison of phage lytic activity by Multiplicity of Infection

Inhibition of *A. hydrophila* growth by the three bacteriophages was compared using MOIs of 10, 1, and 0.1. The phage titers at 24h and 48h are presented in Table 2.3. The optimum MOI of 1, 0.1, and 0.1 yielded higher titers in Ahyd1, Ahyd2, and Ahyd3, respectively.

Table 2.3. Comparison of phage lytic activity and determination of appropriate MOI

Duration	Phage ID	Phage Titres at 48h for the different MOIs (Average PFU/ml +SD) 10^7			
		10	*1*	*0.1*	*0.01*
	Ahyd1	1.4±0.01	3.3±0.42	1.7±0.01	1.8±0.16
24 Hours	Ahyd2	3.5±0	3.4±0.28	4.2±0.30	3.6±0.01
	Ahyd3	1.3±0.14	2.0±0	2.1±0.1	1.9±0.60
	Ahyd1	6.8±0.84	7.3±0	6.9±0.35	6.9±0
48 Hours	Ahyd2	7.8±0.14	8.5±0.35	9.1±0.28	7.7±0.14
	Ahyd3	5.9±0	6.1±0.28	6.8±0.30	6.4±0.29

Thermostability of the Bacteriophage

Thermal stability studies revealed that all the tested phages were stable at temperature ranges of 40–50°C with a slight decrease at 60°C after exposure for 60 min (as shown in Figures 2.3a, 2.3b, and 2.3c). Phage viability was significantly ($P < 0.05$) affected at higher temperatures of

70°C and 90°C (as shown in Figures 2.3a, 2.3b, and 2.3c). At 90°C, no infectivity was detected after 10 min of incubation.

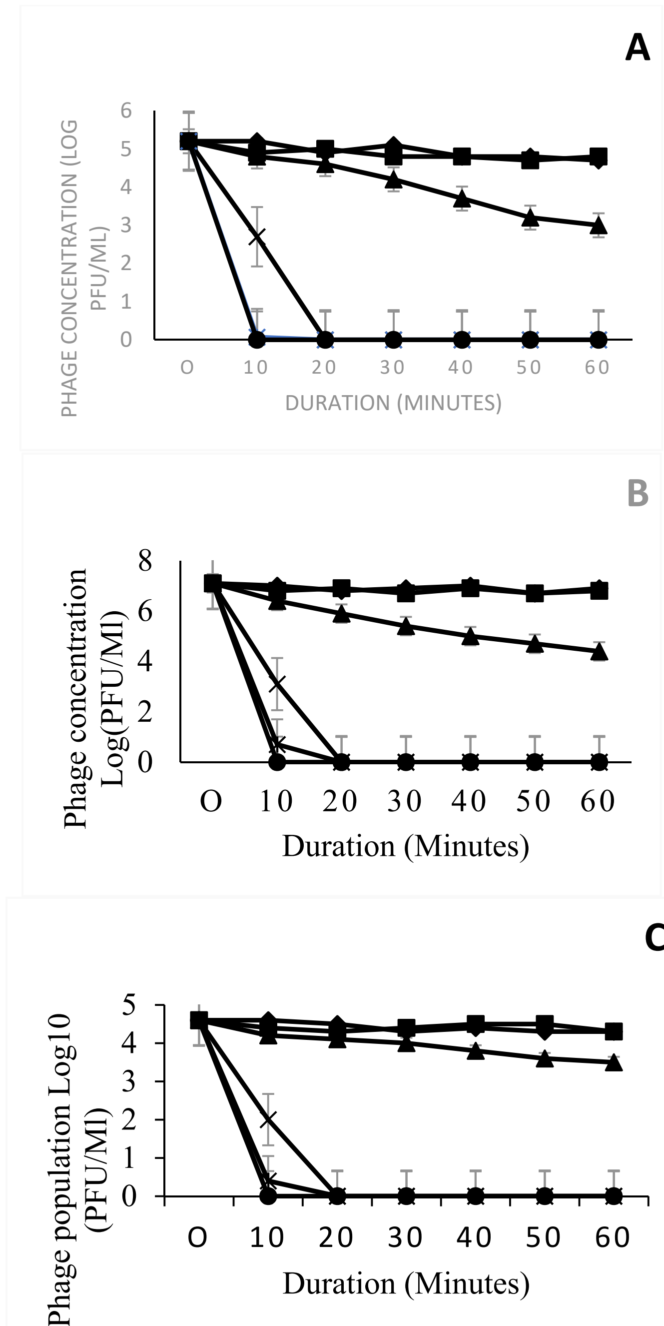

Figure 2.3. Effect of temperature on bacteriophage titers (A) Phage Ahyd1 (B) Phage Ahyd2 and (C) Ahyd3

40 ℃ 50 ℃ 60 ℃ 70 ℃ 80 ℃ 90 ℃

Phage stability under cold storage conditions

Phages were found to be more stable at refrigeration temperature (4℃) than at freezing temperatures of −20℃ throughout the 90 days. At 4℃, minimal reduction in phage titers was observed during the 90 days. All phages were stable, with minor loss in titers during the 90-day storage (Fig. 2.4A). At −20℃, all the three phage isolates lost a considerable amount of their population, followed by the slight reductions in the following days till the last day of the experiment. The final concentration was above 50% for both bacteriophages isolates (Fig. 2.4B).

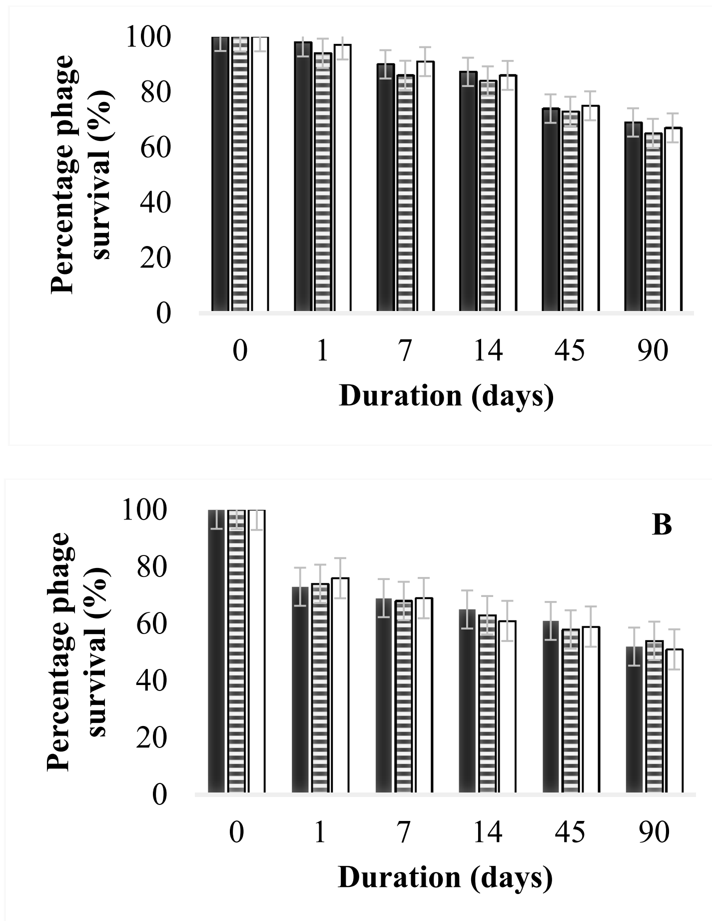

Figure 2.4. Phage stability at 4°C for 90 days (A) and at -20°C for 90 days (B)

■ Ahyd1 ▤ Ahyd2 □ Ahyd3

Discussion, Conclusion and Recommendations

Aeromonas spp are known for being one of the dangerous fish pathogens and have been previously reported to be resistant to the common drugs used in the management of fish diseases. This research revealed that the majority of the *Aeromonas* spp were multi-drug resistant

at 82.6%. Similar results of high multidrug resistance were reported where 100% of *Aeromonas* species were resistant to multiple drugs (Au-Yeung et al., 2022; Puneeth et al., 2022). Despite claims of not applying antibiotics on the fish farms, a high level of the multidrug-resistant *Aeromonas* spp recorded might be because antibiotics supplemented feeds are commercially available without the farmers' knowledge. Alternatively, the runoffs of antibiotics used in terrestrial animal production systems in the vicinity or in case of mixed farming might result in selection pressure. Antimicrobial drugs used in aquaculture are usually administered in feed, either compounded during manufacturing or surface-coated onto feed pellets; they are also used in the hatchery as bath medication. Besides, the discharge of untreated waste onto land and water bodies has also exposed aquatic animals to low doses of antimicrobial agents (Watts et al., 2017). Which may have contributed to the resistance pattern observed in this study in some way.

In this study, all the isolates were sensitive to Ciprofloxacin and Gentamycin, which is similar to findings by (Dahanayake et al., 2020). This might be because the two drugs are rarely used in aquaculture, therefore the bacteria isolates were not exposed to generate resistance. The resistance to uncommon drugs like Chloramphenicol in aquaculture was not expected this might be due to the illegal use of banned antibiotics in aquaculture, same findings were earlier reported (Ng et al., 2014). Medium sensitivity was exhibited for Penicillin, Tetracycline, Nalidixic acid, Nitrofurantoin, Streptomycin, and Trimethoprim/ Sulphamethoxazole, similar to what was recently reported by (Deng et al., 2014; Zhou et al., 2019). This might be due to antibiotic-supplemented fish feeds (Zhang et al., 2020), however, there is no evidence of such existing in Uganda. All the isolates were resistant to Ampicillin because aeromonads are intrinsically resistant to the drug. Indeed high level of Ampicillin resistance among the *Aeromonas* spp was reported by previous researchers (Walsh et al., 1997). The intrinsic resistance is the reason for its use as a selective agent during isolation of aeromonads (Huddleston et al., 2007). Ampicillin-supplemented media were employed for isolation of *Aeromonas* spp from shrimp, fish, and bivalves by (Odeyemi & Ahmad, 2017).

For host range, all *Aeromonas* spp were susceptible to phage Ahyd1 while three isolates showed resistance to Ahyd2, and one showed resistance to Ahyd3. An earlier study, where 40.1% sensitivity of *Aeromonas* spp to the bacteriophage, which differs from the findings of this study, was reported and it is probably due to bacteriophage resistance (Fukuyama *et al.*, 1991). Although the three best phages had wide host ranges, the use of a bacteriophage cocktail is the best option to fight against possible resistance, since their combined activity cleared all the *Aeromonas* isolates tested. Some recent studies reported an improved combined effect of the Aeromonas bacteriophage cocktail (Cheng et al., 2021). Inter-species phage host specificity was done against tentatively confirmed *Edwardisella tarda* and *Klebsiella* spp. At least one phage had cleared one of the three *Edwadisella tarda* and two *Klebsiella* spp. the ability to lyse bacteria from other genera of bacteria that are common fish pathogens is of an advantage to develop phage cocktails that can eliminate fish pathogens together. However, few *Klebsiella* spp and *Edwardsiella* spp were tested, there is need for a more comprehensive analysis.

The comparison of phage infectivity by the multiplicity of infection for the bacteriophages Ahyd1, Ahyd2, and Ahyd3 indicated the optimum MOI as 1, 0.1, and 0.1, respectively. Recent studies suggested that MOI was one of the important parameter when determining for dosage on phage therapy application (Akmal *et al.*, 2020). These optimum MOI for the selected phage isolates could therefore be applied during development of products for control strategies of the transmission and survival of *Aeromonas* spp on fish farms (Easwaran et al., 2017).

Bacteriophage efficiency to infect bacterial cells was measured using a one-step growth curve. Isolated phages had a burst period (20–40 minutes) and a latent period (10–40 minutes) with a

burst size 98–171 virions per infecting cell. Burst size of Aeromonas bacteriophage of 139 virions per infected cell, were reported earlier (Akmal *et al*., 2020). Phage burst size, time and latent period are highly influenced by the host-virus specificity. The latter supports the differences noted for the three phages, where Ahyd1 had the largest burst size of 171 virions per infected cell, which was much higher than other Ahyd2 and Ahyd3. Considering the three parameters determined with the one-step growth experiments, Ahyd3 was the best candidate for phage therapy since it exhibited a short latent period and large burst size. A phage with a short latent period has a selective advantage over others in therapy due to high and fast lytic activity (Park et al., 2012).

Temperature is one of the strong factors that affect the multiplication of bacteriophages. In this study bacteriophages Ahyd1, Ahyd2, and Ahyd3 had the same growth patterns. The phages were stable up to the temperature of 50℃, results obtained by Akmal *et al* (2020) showed that the stability of phage was up to 46℃, this variation might be due to the differences in the temperature intervals during the experiment, and the phage strains. At 60℃ there was a noticeable reduction with a gradual constant decrease in bacteriophage population, then the concentration was negatively affected at 70℃ within 20 minutes the bacteriophage concentration was not viable, while on 90℃ it only took 10 minutes, same conclusions were made in a recent study that the phages concentration were unstable at higher temperatures, this might be due to proteins (viral particles) degradation and denaturation (Akmal *et al*., 2020).

All phages showed that 4℃ storage was more efficient in recovering the higher titer of bacteriophages. A similar conclusion has been drawn by some researchers although their titer reductions were lower compared to this study, which could have been due to repetitive freezing and thawing (Rai et al., 2019). The abrupt reduction of concentration after 24 hours (one day) storage of bacteriophage was observed probably due to cold shock, although after the first day of storage the percentage reduction in the concentration of the bacteriophage increased after each of the following days.

The antibiotic susceptibility testing revealed a high level (82.6%) of multidrug-resistant isolates among the *Aeromonas* spp from the isolates tested. Bacteriophages isolated in this study showed a wide host range along with high lytic activity and thermal stability, and could therefore be used as bio control agents in the management of *Aeromonas* contamination or infections on fish farms. This information will afford the ability to determine the application of phage-based interventions. It is recommended that:

- The safety of phages needs to be determined to rule out presence of virulence or resistance genes before product development for phage therapy.
- Electron microscope should be used for the bacteriophage isolates to establish a proper classification of each isolate basing on their morphologies.
- More comprehensive studies should be done on the physico-chemical characterization of the bacteriophage, e.g., effect of pH, cations, proteolytic and other enzymes on phage stability.
- More studies against a large number of the different fish pathogens as well as normal microbiota is recommended, to confirm the specificity of the three phage isolates.

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