Isolation and Characterization of Multidrug Resistance *Aeromonas salmonicida* subsp. *salmonicida* and Its Infecting Novel Phage ASP-1 from Goldfish (*Carassius auratus*)

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Abstract In this study, *Aeromonas salmonicida* subsp. *salmonicida* was isolated, identified by 16S RNA sequencing and its potential lytic phage (ASP-1) was isolated and characterized. The bacterium was positive for virulence genes (*ascV*, *fla*, *ahyB*, *gcaT*, *lip*, *alt* and *act*) and phenotypic parameters (haemolysis, slime production, lipase activity, DNase test, gelatinase activity and protease activity) were tested. The bacterium was resistant to 27%, intermediate resistant to 14% and susceptible to 59% of tested common antibiotics. Transmission electron microscopy analysis revealed that lytic ASP-1 belongs to the *Myoviridae* family. The isolated phage was more specific against *A. salmonicida* subsp. *salmonicida* (efficiency of plating index = 1), but also had infectivity to *A. hydrophila* lab strain 1. The bacteriolytic effect of ASP-1 was tested at early exponential phase culture of *A. salmonicida* subsp.

salmonicida, and bacteria growth was apparently decreased with time and MOI dependent manner. One-step growth of ASP-1 showed approximately 30 min of latent period, 16 PFU/infected cells of burst size and 40 min of rise period. The adsorption rate was determined as 3.61×10^8 - PFU mL⁻¹ min⁻¹ for 3 min, and rate decreased with time. The ASP-1 genome size was estimated to be approximately 55–60 kD. The phage was stable over wide-range of temperatures, pH and salinity, thus could withstand at severe environmental conditions, indicating that ASP-1 has a potential to develop as an alternative antibiotic to use in ornamental and aquaculture industry.

Keywords *Aeromonas salmonicida* · *Aeromonas salmonicida* subsp. *salmonicida* · Biocontrol · Lytic phage · Pathogenicity · Phage infectivity

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Introduction

The genus *Aeromonas* sp. are autochthonous microbiota found in sewage, brackish water, freshwater and even in drinking water [1, 2] and considered to be an important disease-causing pathogen of fish and other warm and cold-blooded animals [3]. They also act as the etiological agent responsible for causing various infections in both immunocompromised and immunocompetent people [2]. *Aeromonas salmonicida* subsp. *salmonicida* is widely spread pathogen which affect to wild and cultured fish, specially causes severe effects on salmonid populations [4–8], and also has been isolated from non-salmonid fresh and marine water species [9–11]. Mainly, the disease cause via skin lesions, gills and epithelium of the intestine and causes hemorrhagic septicaemia, soft tissue rot, fin rot and furunculosis disease [8, 12].

Producers and retailers in ornamental industry use antibiotics to control or prevent bacterial diseases. Generally, depending on the type of *Aeromonas* or severity of the infection, prescribed antibiotics are either added to water or ingested to the fish by incorporating to the feed. However, the excessive use of antibiotics increases the risk of acquisition and spreading antimicrobial-resistant bacteria [13–15]. Thus, in aquaculture and ornamental fish industry, the use of antimicrobials to control and prevent *Aeromonas* infection need to be carefully monitored and effective alternatives need to be suggested.

Phages infecting *Aeromonadaceae* have been isolated and characterized from different environments [16–20]. Reports demonstrated that phage efficacy could vary with type and effectiveness of the bacterial disease and the fish species. For instance, *Aeromonas* phages have been used to treat furunculosis in brook trout (*Salvelinus fontinalis*) [21], Atlantic salmon (*Salmo salar*) [22], rainbow trout (*Oncorhynchus mykiss* Walbaum) [23], and Senegalese sole (*Solea senegalensis*) [24]. However, control of furunculosis by bacteriophage was less promising for the Atlantic salmon when it challenged with *A. salmonicida* subsp. *salmonicida* [22], even though, phage administered by in vivo did not show any adverse effects. Thus, it is important to continuously investigate new *Aeromonas* phage targets and their characteristics, related to host specificity and growth attributes, to make them as potential therapeutic candidates for controlling pathogenic bacteria in ornamental and aquaculture industry.

In the present study, we describe the *A. salmonicida* subsp. *salmonicida*, isolated from diseased gold fish with respect to identification by 16S RNA sequencing, virulence genes, phenotypic pathogenicity parameters as well as resistant and susceptibility to antimicrobials that commonly use in aquaculture. Phage ASP-1 which is having lytic efficiency to isolated multidrug resistance *Aeromonas salmonicida* subsp. *salmonicida* was isolated and further characterized to find the potential for controlling the *A. salmonicida* infection in ornamental and aquaculture industry.

Materials and Methods

A. salmonicida Strain, Media and Growth Conditions

A. salmonicida strain was isolated from gold fish, purchased from Daejeon aquarium, Republic of Korea. The fish appeared to be sick with weak, lethargic and disoriented swimming behavior. Fish kidney, gut and skin tissues were aseptically removed, homogenized and decimally diluted in phosphate buffered saline (PBS). Hundred micro

liter was plated on tryptic soy agar (TSA; pH 7.2) followed by an overnight incubation at 25 °C. The obtained colonies were further screened to acquire pure colonies with uniform morphology, which were identified by 16S rRNA sequencing (Cosmogenetech co. Ltd., Republic of Korea) using universal 27F-forward primer (5' AGAGTTT-GATCMTGGCTCAG 3') and 1492R-reverse primers (5' TACGGYTACCTTGTTACGACTT 3'). The isolated *A. salmonicida* subsp. *salmonicida* strain was stored in TSB medium with 50% glycerol stock at -80 °C.

Isolation of *A. salmonicida* subsp. *salmonicida* Genomic DNA and Screening of Virulence Factor-Encoding Genes by Polymerase Chain Reaction (PCR)

Genomic DNA isolation was carried out using a commercial kit (Bioneer, Korea) following the manufacturer's recommendations. Eleven virulence factor encoding genes *ascV*, *fla*, *ahyB*, *gcaT*, *exu*, *ser*, *lip*, *aerA*, *ast*, *alt* and *act* were amplified by PCR (Supplementary Table 1). The PCR reaction mix was made in 25 µL volume (2.5 µL of 10× *Ex Taq* buffer, 2.5 µL of dNTP (2.5 mM), 0.25 µL of *TaKaRa Ex Taq*TM polymerase (5 units/µL), 2.5 µL of each reverse and forward primer (1 µM final concentration), 2 µL of template DNA (200 ng) and 12.75 µL of PCR water). The thermal cycling consisted of an initial denaturation of 94 °C for 2 min followed by 35 cycles of amplification. Each cycle consisted of 94 °C denaturation phase for 30 s, annealing phase temperatures as described in supplementary Table 1 for 50 s, 1 min elongation at 72 °C and final extension at 72 °C for 10 min. Amplified PCR products were resolved on 1.5% agarose gels stained with RedSafeTM nucleic acid staining solution (iNtRON Biotechnology, USA).

Phenotypic pathogenicity tests

The phenotypic pathogenicity of *A. salmonicida* subsp. *salmonicida* was evaluated by assaying hemolysis on sheep blood agar (MB Cell. LA, CA) plates [29], slime production on TSA (MB Cell. LA, CA) supplemented with 5% sucrose and 0.08% Congo red (w/v) [30], lipase activity on TSA plates supplemented with 1% Tween 80 (v/v) [31], DNase activity on DNase test agar (MB Cell. LA, CA) [32] and gelatinase activity on gelatin medium added with 5% peptone, 3% beef extract and 15% gelatin (w/v) at final pH 7 [33]. Extracellular protease activity was assayed in TSA supplemented with 1% casein (v/v) [34].

Antibiotic Resistance and Susceptibility Analysis of *A. salmonicida* subsp. *salmonicida*

Disc diffusion assay was conducted for 22 antimicrobials belonging to 12 antimicrobial classes (Table 1) using BD BBL™ Sensi-Disc™ antimicrobial susceptibility test discs (Becton, Dickinson and Company, USA). Four antibiotic discs per plate were placed on *A. salmonicida* subsp. *salmonicida* spreaded TSA plates under aseptic conditions and incubated at 25 °C for 24 h. The diameter of the inhibition zone was measured around the Sensi-Disc (6 mm).

Isolation of *A. salmonicida* phage (ASP-1) Specific to *A. salmonicida* subsp. *salmonicida*

The phage ASP-1 was isolated following the standard enrichment procedure using above mentioned *A. salmonicida* subsp. *salmonicida*. Chopped gold fish tissues (kidney and digestive tract) were mixed with 25 mL 2× TSB and overnight grown *A. salmonicida* (OD₅₉₅; 0.2 = 2.8×10^7 CFU/mL), and incubated with shaking at 25 °C for 24 h. The enriched sample was centrifuged at

7000 g for 20 min at 4 °C. The supernatant was filtered through a 0.2 µm membrane filter (Minisart syringe filter, Germany), and the presence of lytic phages in the supernatant was detected by a standard double-layer agar overlay method [35]. The obtained phage plaques were purified to a single morphology plaque by repeatedly picking, eluting in SM buffer (pH 7.5) (50 mM Tris HCl; pH 7.4, 0.1 mM NaCl, 8 mM MgSO₄) and plating thrice. Out of different morphology of single plaques, *A. salmonicida* phage ASP-1 was chosen based on their ability to perform bacteriolytic effects. The chosen plate lysate was further purified by polyethanol glycol/NaCl (PEG/NaCl) precipitation followed by filtration and dialysis. The ASP-1 titer (PFU mL⁻¹) was determined by standard double layer agar method. The purified phage was stored in SM buffer at 4 °C.

Transmission Electron Microscopy (TEM) Analysis of ASP-1

Dialysed CsCl-purified phage stock was used for transmission electron microscopy (Bio-TEM; Hitachi, Tokyo, Japan) under acceleration voltage of 100 kv and 200,000 ×

Table 1 Antimicrobial resistance and susceptibility of *A. salmonicida* subsp. *salmonicida*

Antibiotic class	Antimicrobials	Disc content (µg)	Inhibition zone diameter ^a
Penicillins	Ampicillin	10	0 (R)
	Amoxicillin 1/clavulanic acid	20/10	8 (R)
Cephalosporins	Cephalothin	30	13 (R)
	Cefotaxime	30	30 (S)
	Cefoxitin	30	26 (S)
	Ceftriaxone	30	36 (S)
	Streptomycin	10	0 (R)
Aminoglycosides	Amikacin	30	15 (I)
	Tobramycin	10	10 (R)
	Gentamycin	10	20 (S)
	Kanamycin	30	22 (S)
	Vancomycin	30	0 (R)
Glycopeptides	Erythromycin	15	19 (I)
Macrolides	Clindamycin	5	16 (I)
Lincosamides	Tetracycline	30	28 (S)
Tetracyclines	Chloramphenicol	30	25 (S)
Phenicol	Nalidixic acid	30	28 (S)
Quinolones	Ciprofloxacin	5	30 (S)
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	25	23 (S)
Fluoroquinolone	Levofloxacin	5	30 (S)
	Norfloxacin	10	29 (S)
Monobactams	Aztreonam	30	28 (S)

S = susceptible; I = intermediate; R = resistant were designated using breakpoints described by the BBL™ Sensi-Disc™ Zone Interpretation Chart (Becton, Dickinson and Company, USA)

magnification, after subjected to negative staining with 2% uranyl acetate [36].

Determination of ASP-1 Host Range and Efficiency of Plating (EOP)

The host range of ASP-1 was determined for 36 bacterial strains. In Genus *Aeromonas*, the bacteria used were *A. salmonicida* strains (04) including *A. salmonicida* subsp. *salmonicida* (indicator host), *A. hydrophila* strains (06) including *A. hydrophila* (Lab strain 1), *A. sobria* (01), *A. bivalvia* (01), *A. caviae* (01) and *A. veroni* (02). Apart from those, *Morgenella* sp. (02), *Edwardsiella tarda* (03), *Enterobacter* sp. (01), *Enterococcus faecium* (01), *Escherichia coli* (01), *Klebsiella* sp. (02), *Streptococcus iniae* (02), *S. parauberis* (03), *Vibrio* sp. (05), *Lactococcus* sp. (01) were also tested for host specificity. The spot test [36], was considered as positive for clear zones or single plaques on the bacteria lawn. The lysed, slightly disrupted, or not disrupted bacteria on the lawns were marked as sensitive, intermediate, or resistant to the phage infection, respectively. The EOP was calculated using the formula; EOP index = [Number of plaques per mL for the specific host/Number of plaques per mL for the indicator host]. EOP index > 1, < 1 and = 1 was considered as high, low and with similar efficiency to the indicator host, respectively.

Inhibition of *A. salmonicida* subsp. *salmonicida* Growth by ASP-1

Log phase cultures of the *A. salmonicida* ($OD_{595} = 0.188$; 1.0×10^7 CFU mL⁻¹) was mixed with ASP-1 (3.7×10^{11} PFU mL⁻¹) at a multiplicity of infection (MOI) of 10, 1, 0.1 and 0.01. Mixtures were incubated at room temperature (RT; 22 ± 3) for 20 min for phage adsorption, and further incubated in a shaker at 25 °C. Changes in the bacterial growth over time were monitored by measuring the OD at 595. Equal volume of SM buffer was mixed with log phase bacterial cultures under same experimental conditions to serve as a negative control.

Determination of Latent Period and Burst Size of ASP-1

The latent period and burst size of ASP-1 was determined by one-step growth curve. ASP-1 was mixed to *A. salmonicida* culture ($OD_{595} = 0.188$) at MOI 1 in 20 mL of fresh TSB at RT for 12 min, and centrifuged at 7000 g for 5 min. Obtained pellet was slowly re-suspended in 20 mL fresh TSB, and 1 mL of each was aliquoted into 12 Eppendorf tubes. One tube at a time was taken at 10 min interval from 0 to 90 min, and centrifuged immediately at 13,000 g. Supernatant was serial diluted (up to 10^5

in SM buffer), and performed standard double-layer agar overlay method to count the phage titer at each time point.

Determination of ASP-1 Adsorption Efficiency and ASP-1 Adsorption Rate Constant

The adsorption rate of ASP-1 was estimated at MOI 1 with the *A. salmonicida* subsp. *salmonicida* culture ($OD_{595} = 0.188$). The bacteria culture was treated with ASP-1 at RT, and from the mixture, 100 µL samples were removed at 0, 3, 6, 9, 12, 15, 18, 25, 30 and 35 min and immediately diluted in 900 µL of SM buffer with chloroform (45 µL mL⁻¹). Samples were vigorously vortexed and centrifuged at 13,000 g for 2 min at RT. The number of free phages in the supernatant was determined as described previously. The phage adsorption rate and phage adsorption rate constant (*K*) was calculated according to Stenholm et al. [37] and Barry and Goebel [38].

ASP-1 Stability Assays

The sensitivity of ASP-1 was investigated under different temperatures (4–90 °C), pH values (2–14), salinities (0.1–3.5%) and organic solvents including diethyl ether: (CH₃CH₂)₂O, chloroform: CHCl₃, acetone: (CH₃)COCH₃ and ethanol (CH₃CH₂OH). For each test, 100 µL of phage was inoculated in 900 µL of SM buffer or respective solvent, incubated at 1 h under given conditions and surviving phages were enumerated using standard double-layer agar overlay method described elsewhere. In each test, stability percentage was calculated based on the highest surviving phage number. The data were analysed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism software version 6 for windows (GraphPad Software Inc. USA). The differences were considered statistically significant at $p < 0.05$ and data were presented as mean $\pm$ standard error of mean.

Isolation of ASP-1 Genomic DNA and Restriction Digestion Analysis

The genomic DNA was isolated using purified phage stock (3×10^{11} PFU mL⁻¹) using GeneAll[®] Exgene[™] Cell SV DNA extraction kit (GeneAll Biotechnology, Co., Ltd., Rep. of Korea), and restriction digested with EcoRI, EcoRV, XhoI, SphI, HindIII, NdeI, SpeI, ClaI, KpnI and SphI (TaKaRa, Japan). The digested samples were then resolved in 1% agarose gel by electrophoresis at 50 V for 30 min. Genome size was predicted based on the band pattern of ClaI digested phage genomic DNA, with respect to the 1 kb molecular size marker (Bioneer, Rep. of Korea) and Lambda DNA/HindIII marker (Promega, USA).

Results

Isolation and Identification of *A. salmonicida* subsp. *salmonicida* Strain

Several rounds after bacterial screening from the gold fish tissues, the bacterium was identified by 16S rRNA sequencing analysis. It was identified up to sub species level as *A. salmonicida* subsp. *salmonicida*, and had 100% identity with *A. salmonicida* subsp. *salmonicida* strain AS01; Accession no. MG384968.1 of nucleotide BLAST search results obtained from National Center for Biotechnology Information (NCBI) database.

Determination of Pathogenicity and Virulence Factors Encoding Genes of *A. salmonicida* subsp. *salmonicida*

For all the tested pathogenicity assays (haemolysis, slime production, lipase activity, DNase test, gelatinase activity, protease activity), *A. salmonicida* subsp. *salmonicida* isolate showed positive. Moreover, among eleven tested virulence genes, seven were positive (*ascV*, *fla*, *ahyB*, *gcaT*, *lip*, *alt* and *act*) and four were negative (*exu*, *ser*, *aerA* and *ast*) (Supplementary Figure 1).

Determination of Antimicrobial Resistance/ Susceptibility

Isolated *A. salmonicida* subsp. *salmonicida* showed 59% (13/22) of susceptible, 27% (6/22) of resistant (ampicillin, amoxicillin 1/clavulanic acid, cephalothin, streptomycin, tobramycin, vancomycin), 14% (3/22) of intermediate resistant (amikacin, erythromycin and clindamycin) to antibiotics tested (Table 1).

Isolation and Morphology of Phage ASP-1

Phage ASP-1 formed a clear plaque with a diameter of 1–2 mm (Fig. 1a), and the titer that maximally observed was 10^{12} PFU mL⁻¹. Figure 1b, c show the TEM image of ASP-1. Morphological analysis of TEM revealed that ASP-1 has an icosahedral head (58.51 ± 1.21 nm, $n = 6$) and a contractile tail (157.23 ± 2.43 nm, $n = 6$ long and 19.76 ± 1.02 nm, $n = 6$ wide).

ASP-1 Host Range Determination and EOP Analysis

ASP-1 infects mainly its indicator host *A. salmonicida* subsp. *salmonicida* (EOP index = 1) without infecting other *A. salmonicida* strains (03) tested. ASP-1 was also able to infect *A. hydrophila* (Lab strain 1), however, its

EOP index showed negligible value ($1 \times 10^{-5} \pm 1 \times 10^{-6}$). The phage infects to none of the bacteria species belong to other nine genera.

Determination of *A. salmonicida* subsp. *salmonicida* growth inhibition by ASP-1

The in vitro growth inhibition by ASP-1 is shown in Fig. 2 at different MOIs. The clear growth inhibition was observed by ASP-1 compared to the control bacteria culture. The time and MOI dependant lytic activity was demonstrated, showing the highest MOI at 10, yielding a more rapid decline of *A. salmonicida* subsp. *salmonicida* population. The bacteria population was started to decrease from 1 h at 10 MOI of phage addition, while it was started to decrease at 2 h at MOI 1 and 0.1 MOI, respectively. This low level of bacterial population was maintained until 12 h at all these MOIs, whereas it took 6 h to reach same level of bacterial population when phage added at MOI of 0.01. However, at approximately 12 h post inoculation of ASP-1, bacteria OD₅₉₅ values started to increase.

Determination of the ASP-1 Adsorption Rate and One-Step Growth Profile

As shown in Fig. 3a, free phages in the supernatant were decreased slowly. It took approximately 25 min to decrease 70% of free phages (unadsorbed phage) in the supernatant. Figure 3b shows the adsorption rate of ASP-1 from 3 to 25 min after bacteria and phage mixed. The rate of asorption was quicker at 3 min followed by 6 and 9 min compared to rest of the time points. The adsorption rate of ASP-1 at 3 min was 3.61×10^8 PFU mL⁻¹ min⁻¹, and it was 6.1×10^7 PFU mL⁻¹ min⁻¹ for 25 min. The rate constant for the adsorption (loss of free phages), K was 8.30×10^{-9} mL min⁻¹. The one-step growth curve exhibited a latent period of 30 min, long rising period of 40 min with a burst size of 16 phage PFU per infected cell (Fig. 3c).

Determination of ASP-1 Genome Size

Supplementary Figure 2 shows the ASP-1 genomic DNA subjected to restriction cut by ClaI and SphI. The ClaI showed different sizes of digested DNA fragments, whereas SphI exhibited smear DNA. The rest of the tested restriction enzymes did not digest ASP-1 genomic DNA. Based on the ClaI digestion pattern, the size of ASP-1 genome was estimated as 55–60 kb.

Fig. 1 Isolation of *A. salmonicida* phage (ASP-1) and TEM image of ASP-1. **a** Double laid TSA soft agar plate showing plaque morphology of ASP-1 infecting *A. salmonicida* subsp. *salmonicida*. **b** TEM analysis showing icosahedral head and sheathed tail tube with tail

fiber of ASP-1 that negatively stained with 2% uranyl acetate. Bar = 100 nm. **c** TEM analysis showing ASP-1 contractile tail of ASP-1. Bar = 100 nm

Fig. 2 Time course bacteriolytic activity of ASP-1. Phage was added at different multiplicity of infections (MOIs; 10, 1, 0.1, 0.01) to early log-phase grown *A. salmonicida* subsp. *salmonicida* as described in materials and methods. As a control, autoclaved SM buffer was inoculated with *A. salmonicida* subsp. *salmonicida*. The data represent results from three independent experiments. Error bars indicate the $\pm$ SE of means at each time point

ASP-1 Stability Assays

ASP-1 survival was stable over wide ranges of temperatures (4–50 °C) ($p < 0.05$), pH (4–11) ($p < 0.05$) and salinity (0.1–3.5%) ($p > 0.05$) as shown in Fig. 4a–c, respectively. The temperature stability was significantly different at 4, 25 and 50 °C compared 65 °C, while phage was not survived at 65 °C. Phage stability was more than 95% at 4, 25 and 50 °C, however, at 37 °C, the survival was varied ($50 \pm 15\%$), but was not significant. The phage survival was totally lost at pH 2, 3, 12, 14 and the highest pH stability was observed at pH 11. As shown in Fig. 4d, survival results with organic solvents were significantly different ($p < 0.05$). It demonstrated greater stability for chloroform, PBS and SM buffer (showed almost 100% survival) followed by diethylether (for 1 h showed approximately $80 \pm 22\%$ stability). However, infectivity was completely lost in ethanol and acetone. The optimal

Fig. 3 *In vitro* ASP-1 growth characterization. **a** Free phages in the ASP-1 and *A. salmonicida* subsp. *salmonicida* supernatant. **b** Adsorption rate of ASP-1. Adsorption rate values were plotted for each time point from 3 to 35 min. **c** One-step growth curve of ASP-1. All the experiments were conducted at MOI = 1. Error bars represent the $\pm$ SE of the means ($n = 3$)

Fig. 4 Stability tests of ASP-1. Stability analysis of ASP-1 under **a** temperature (4–65 °C), **b** pH (2–14), **c** salinity (0.1–3.5%), and **d** organic solvents (chloroform, ethanol, diethylether, acetone). Phage stability percentages of temperature, pH, salinity and organic

solvents were calculated based on 4 °C, 11, 1% and PBS, respectively. Data represent the results of two independent experiments. Error bars represent the $\pm$ SE of the means

temperature, pH and salinity resulting the highest phage stabilities observed at 4 °C, 11 and 1%, respectively.

Discussion

In this study, isolated *A. salmonicida* subsp. *salmonicida* was tested for its pathogenicity, virulence characteristics, and antibiotic resistance properties against commercially use common antibiotics. Virulence of *Aeromonas* sp. is multifactorial, and several virulence factors related to colonization, proliferation and immune evasion are associated with the pathogenic potential of the *Aeromonas* sp. in the host [25–28, 39, 40]. It has been reported that *ser* and *gcaT* play a major role in furunculosis caused by *A. salmonicida* [40], and Lago et al. [41] reported that both virulent and a virulent *A. salmonicida* subsp. *salmonicida* (ASS) strains isolated from turbot possessed all the virulence factors related genes that they tested. However, virulence gene distribution in *Aeromonas* largely varies among species of the genus, and also the strains of the same species and source of isolation, thus vary the virulence [26, 27, 42]. In this study, out of 11 virulence genes tested, *ascV*, *fla*, *ahyB*, *gcaT*, *lip*, *alt* and *act* were found to

be present, whereas 4 genes; *exu*, *ser*, *aerA* and *ast* were absent. Even with their absence, the presence of multiple virulence factors together with positive results for the phenotypic characteristics suggest that isolated *A. salmonicida* subsp. *salmonicida* have potential to be causing pathogenicity. However, it is important to consider not only the presence of virulence genes, but factors such as host susceptibility for the infection and environmental changes that cause infections.

Most of the *Aeromonas* strains isolated from gold fish have been resistant, especially to β -lactams (e.g. amoxicillin, ampicillin and cephalothin), tetracycline, trimethoprim/sulfamethoxazole, nalidixic acid and doxycycline [43]. In this study, the results of the disc diffusion assay indicates that the isolated *A. salmonicida* subsp. *salmonicida* consists of multiple antimicrobial resistance, and there is a possibility to decrease the susceptibility to the antibiotics by frequent use.

Previously, several phages for *A. salmonicida* strains [44] and *A. salmonicida* subsp. *salmonicida* have been isolated and characterized [18, 45]. As a potential alternative to antimicrobials, we isolated and characterized the lytic phage ASP-1, which mainly infects *A. salmonicida* subsp. *salmonicida*. Le et al. [20] reported that most of the

Aeromonas phages isolated are belonging to family *Myoviridae* and in natural environments, members of the family *Myoviridae* are most likely to be abundant. Morphological analysis of ASP-1 by TEM revealed features similar to typical characteristics of members of the family *Myoviridae* with long contractile tail and isometric head [46]. With the tail contraction, tail sheath become loose and slides along the tail core. Under contracted state, tail fiber structures attached to the baseplate become clearer (Fig. 1c). The dimensions of the head and tail structures of ASP-1 lies within the dimension range of other classified *Aeromonas* phages, however, a wide variation can be seen within the family members [16, 47]. Additionally, restriction digestion by *Cla*I of ASP-1 genomic DNA suggests that ASP-1 is a double stranded DNA genome with an estimated genome size of 55–60 kb, however the actual genome size could be larger than this size, since ASP-1 being a *Myoviridae* phage.

The present work demonstrated the strong growth inhibition of ASP-1 against *A. salmonicida* even at a low MOI of 0.01, suggesting that it would be useful in phage therapy at low dosages. However, survived and growing bacteria were observed approximately 12 h post inoculation of ASP-1, probably due to the development of phage resistance. Silva et al. [24] reported that AS-A phage had similar resistant phage development, and this phage-resistant mutant did not negatively affect the in vivo studies in controlling *A. salmonicida* infection. Furthermore, Le et al. [20] indicated that for higher MOI values of phage, the development of phage-resistant bacterial cells appeared quickly. Similar observation has reported by Kim et al. [17] for phage PAS 1 against an *Aeromonas salmonicida* strain, that bacterial resistance had shown after 3, 6, and 24 h at MOIs of 10, 1, and 0.1, respectively. As suggested by several researches, developing a cocktail combining two or multiple different phages would be an effective solution to overcome the development of ASP-1 phage-resistant bacterial mutants and broaden the host range [19, 48, 49].

The faster adsorption and relatively low latent periods are beneficial characteristics for a phage when it is used as a therapeutic agent [50]. These values have been shown to vary from low to high between phages. It has been shown that *A. salmonicida* subsp. *salmonicida* strains infecting T4-like bacteriophage phiAS5 [45] and phiAS4 [18] had burst size of 68.1 PFU/cell and 395.8 PFU/cell, respectively with approximately 30 min of latent period. In this study, though the similar latent period was observed, ASP-1 had low burst size of 16 PFU per infected cell, indicating a phage to phage variation during multiplication process. Compatible to our observations, Duarte et al. [44] reported low burst size of 22, 5, and 10 PFU/cells for *A. salmonicida* infecting phages AS-A, AS-D and AS-E at a latent period

of 30, 40 and 40 min, respectively, and that low burst size did not effect to the growth reduction of *A. salmonicida*.

In natural environment, organisms are exposed to different physical and chemical conditions. Thus, for the development of effective phage treatments, studying the stability or survival of phages under different physico-chemical conditions is important [44]. Though the deviated results in stability or survival were reported [44, 45], our results clearly show the ability of ASP-1 to survive in various pH, temperatures and salinities. Especially, as ASP-1 is heat resistant up to 50 °C, it could be beneficial in the medicated feed manufacturing process in aquaculture where high temperatures are required. Furthermore, isolated *A. salmonicida* subsp. *salmonicida* being the main host strain for ASP-1, it also showed low activity against *A. hydrophila* strain that previously isolated from our zebra-fish system [42]. Some reported phages infect only its indicator host [24, 44], however, several *Aeromonas* phages showed broad infectivity against *Aeromonadaceae* [45]. The narrow host specificity would be advantageous over broad host range specificity at certain cases, because it could be helpful for controlling major pathogenic strain without being causing any detrimental effect to the natural microflora or the animal. These facts imply that ASP-1 is not only useful in controlling propagation of *Aeromonas* sp. in natural harsh environmental conditions, but also important biocontrol agent as an alternative to the antibiotics or as a component of a phage cocktail for specified *A. hydrophila* infection. Overall, our data indicate the possibility of the use of ASP-1 as a therapeutic agent for the treatment of *A. salmonicida* subsp. *salmonicida* associated infections in ornamental and aquaculture industry.

Conclusions

A. salmonicida subsp. *salmonicida* isolate showed virulence factor-encoding genes including *ascV*, *fla*, *ahyB*, *gcaT*, *lip*, *alt* and *act* and multi-drug resistance against commonly used commercial antibiotics. The characterized ASP-1 could be categorized as a novel phage belongs to family *Myoviridae* showing strong growth inhibition against *A. salmonicida* subsp. *salmonicida* even at a low MOI of 0.01 and weak inhibition on *A. hydrophila* (Lab strain 1).

However, more strains of *Aeromonas* sp. are needed to test to draw a firm opinion of host range specificity of ASP-1. In future, genome sequencing will be conducted to study the detail genetic characterizations of unfavourable genes, such as lysogeny-associated, toxin and virulence genes before selecting ASP-1 as an alternative antibiotic therapy.

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Compliance with Ethical Standards

Conflict of interest The authors declared that no conflicts of interest.

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