Scientific Research Publishing

Advances in Microbiology, 2019, 9, 495-506
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

N-Acylhomoserine Lactones (AHLs), QseB/C Gene Detection, Virulence Factors and Antibiotics Resistance of *Aeromonas hydrophila*

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How to cite this paper: Sarkodie, E.K., Zhou, S. and Chu, W. (2019) N-Acylhomoserine Lactones (AHLs), QseB/C Gene Detection, Virulence Factors and Antibiotics Resistance of *Aeromonas* hydrophila. *Advances in Microbiology*, **9**, 495-506.
https://doi.org/10.4236/aim.2019.95030

Received: April 2, 2019
Accepted: May 20, 2019
Published: May 23, 2019

Abstract

The aim of this research was to detect the N-acyl homoserine lactones (AHLs) production and QseB/C gene of *Aeromonas hydrophila*. We analyzed the potentials of these isolates of *Aeromonas hydrophila* in causing biofilm formation, hemolysis, protease, and lipase. The antibiotic susceptibility of the 15 *Aeromonas hydrophila* isolates was also investigated. The detection of AHLs was carried out using the *Chromobacterium violaceum* strain CV026 as biosensors. The isolated strains were tested for the reaction of *C. violaceum* CV026 by cross-streaking on an agar plate. Production of AHLs was determined by the diffusing via the agar plates and the tinge of the biosensor strains. All isolated strains produced AHLs. A polymerase chain reaction (PCR) showed the isolated strains had qseB and qseC genes. Susceptibility tests of *A. hydrophila* isolates were administered against 25 different antibiotic disks representing 12 classes of antibiotics. The strains were highly resistant to β-Lactam with 96.7% showing resistibility, whereas 97.7% susceptibility was found towards Aminoglycoside class of the antibiotic used. 60% showed intermediate resistant to Polypeptide. 100% of the strains showed no resistant to Aminoglycoside, Polypeptide, Monobactam, and Carbapenems class of antibiotics. Each of the isolates was found to be associated with at least one virulent factor. Our results clearly demonstrated that there is a presence of QseB/C genes in *A. hydrophila* and also produces AHLs molecule and virulence factors. The investigated isolates showed the pathogenic potential of *Aeromonas hydrophila* which makes it a serious threat to public health.

Keywords

Aeromonas hydrophila, Antibiotic Susceptibility, Virulence Factors, Biofilm Formation, N-Acyl Homoserine Lactones (AHLs)

1. Introduction

Aeromonads are a group of bacteria with multicellular functioning. The genus *Aeromonas* belongs to *Aeromonadaceae* family [1] and within the *Gammaproteobacteria* subclass. Worldwide, aeromonads are found in freshwater, estuarine, and marine environments. *A. hydrophila* forms part of thirty-two validated species in the genus *Aeromonas* [2] and also typifies the high levels of antimicrobial resistance, resulting in difficulties in treating infections in the aquaculture industry. A wide range of diseases among warm- and cold-blooded animals including fish, amphibians, mammals, and humans are caused by *Aeromonas.* Financial losses in aquaculture due to some diseased fish symptoms including skin hemorrhages, red sores, loss of appetite, dropsy, and fins rot occurs as a result of *Aeromonas* infections [3]. *Aeromonas hydrophila* is associated with gastrointestinal and extraintestinal diseases such as wound infections and septicemia [4]. *A. hydrophila* is gram-negative, facultatively anaerobic, oxidase and catalase positive, fermentative, and mostly motile bacilli and well-known environmental opportunistic pathogens of fish, reptiles, and mammals. The genus *Aeromonas* has been categorized into two major groups: Motile, mesophilic species that cause disease in humans, and Non-motile, psychrophilic species that generally cause disease only in fish. They are deemed as an important *Aeromonas* species, liable for an array of human infectious diseases. The pathogenic virulence factors of *A. hydrophila* include adhesions, cytotoxins, hemolysins, and proteases, and are able to form biofilms and alter gene expression and metabolic pathways under various host environments [5] [6]. Over the years, research on *A. hydrophila* has shown that they stick to hard exteriors and establish biofilms on the glass exteriors, polystyrene, polyvinyl chloride, and stainless steel [7]. Biofilms are considered as the leading root of perpetual infection and drug resistance, largely because of their ability to align with the visceral organs and other material surfaces [8]. The most common AIs are the N-acylhomoserine lactones (AHLs) and have the most studied their signaling mechanisms [9]. N-acylhomoserine lactone (AHL)-based Quorum sensing (QS) system, has been found to be one of the autoinducers that exist in *A. hydrophila* and a cross talk exist between AHLs and QseBC systems in *A. hydrophila* [10]. Autoinducers 1, 2 and 3 (Al-1, Al-2 & AL-3) systems effectively coordinate and influence biofilm formation and maintenance in *Aeromonas hydrophila*. There is an impact of QseB/C on quorum-sensing virulence regulatory system in a clinical isolate of *A. hydrophila* [10]. The widespread use of antimicrobial agents as a control mechanism in human and animal epidemics has resulted in an increase of antimicrobial resistance, not only in pathogenic bacteria but also in commensal and environmental bacteria [11]. Over the years, antibiotics have mostly been used as a therapeutic agent for controlling Aeromonas infections, especially in aquaculture. Very little is known about environmental strains regardless of the extensive report on antibiotic susceptibility of clinical isolates of *Aeromonas* spp. 99% resistant methicillin, rifampicin, bacitracin, and novobiocin were observed in all tested strains of *A. hydrophila*

isolated from fish and prawns in south India [12]. The use of Aeromonads as indicators for the biological effect of domesticated animals and fish cultivating studies can be traced to their ubiquitous characteristics in freshwater and the environment which has also made them naturally susceptible to most antimicrobial agents hence the aptness to develop specific or multitudinous antimicrobial resistance. In this study, we investigated the production of AHLs if any, the presence of QseB/C gene, antimicrobial susceptibility and the presence of virulence factors and biofilm formation potentials in *A. hydrophila.*

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

A total of 15 *Aeromonas* strains were obtained from fish and the aquatic environment within the period of June 2017-July 2018 from four different locations in Jiangsu Province, China and identified by phenotypic testing and 16S rDNA sequencing was selected for this study (Table 1). *Chromobacterium violaceum* CV026, used as the biosensor and *A. hydrophila* strains were grown at 37˚C, in Luria Bertani broth (LB) or on LB agar plates. All strains were verified as aeromonads through a biochemical test and included in this study were the type strains for *Aeromonas hydrophila* (ATCC 7966) Strains were stored at −80˚C until the test was performed and a Single colony was inoculated into LB media and cultured overnight at 37˚C.

Table 1. Bacterial strains used in the study and their source.

Bacteria	Strain codes	Isolation Location	Period of collection
A. hydrophila strains	ATCC 7966		
	JH 25	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 26	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 31	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 33	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 34	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 36	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 37	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 38	Jianhu City, Jiangsu Province	June 2017-July 2018
	JH 39	Jianhu City, Jiangsu Province	June 2017-July 2018
	DH 29	Donghai City, Jiangsu Province	June 2017-July 2018
	DH 41	Donghai City, Jiangsu Province	June 2017-July 2018
	JY 24	JiangYan City, Jiangsu Province	June 2017-July 2018
	JY 25	JiangYan City, Jiangsu Province	June 2017-July 2018
	TZ 16	Taizhon City, Jiangsu Province	June 2017-July 2018

2.2. Detection of AHLs and QseB/C Gene in *A. hydrophila*

Detection of AHLs was achieved using the *C. violaceum* strain CV026 as biosensors [13]. *C. violaceum* CV026 was grown in LB medium at 30˚C supplemented with the antibiotic kanamycin, 50 μg·mL^{-1}. The strains to be tested for the reaction of *C. violaceum* CV026 were cross-streaked on an agar plate. Strains that produced AHLs were able to spread through the agar plates and activated the tinge of the biosensor strains. The *qseBC* genes were amplified by PCR using gDNA of *A. hydrophila* ATCC 7966 as the template and two primers qseBF2/qseBR2 qseCF2/qseCR2 (5'-TGCTCAAGAGCGAGGAGTTTG-3', 5'-CTTCTTGCGCAGGTGGTGAAT-3'), (5'-ATGtively. PCGAGGAGCTGTTCGATGCC-3', 5'-AGCAGCATCTTCTGCAGGGAG-3') PCR was performed with a final volume of 25 μL containing 12.5 μL of 2X Taq PCR Master Mix (2x blue dye). The PCR was performed under similar conditions: Initial denaturation at 94˚C for 4 min and 34 cycles of 30 s at 94˚C, annealing for 30 s at 55˚C, and 60 s at 72˚C followed by final extension at 72˚C for 5 min. The amplified products were then analyzed electrophoretically on a 2% agarose gel containing Gold View. Gels were observed under ultraviolet light illumination and the results digitally recorded.

2.3. Antibiotic Susceptibility

Test on antibiotic susceptibility of *A. hydrophila* isolates was conducted against 25 different antibiotic disks representing 12 classes of antibiotics (Table 2). 100 μL of fresh bacterial solution was poured evenly onto the solid common broth

Table 2. List of antibiotic disks and their concentration.

ANTIBIOTIC	CONCENTRATION	ANTIBIOTIC	CONCENTRATION
Penicillin	10 μg	Teicoplanin	30 μg
Amoxicillin	10 μg	Sulfamethoxazole TMP/SMZ	1.25/23.75 μg
Ampicillin	10 μg	Norfloxacin	10 μg
Piperacillin	10 μg	Ciprofloxacin	5 g
Cephalexin	30 μg	Fosfomycin	200 μg
Cefradine	30 μg	Polymyxin B	300 μg
Cefaclor	30 μg	Tetracycline	30 μg
Cefixime	5 μg	Doxycycline	30 μg
Imipenem	10 μg	Azithromycin	15 μg
		Vancomycin	30 μg
Meropenem	10 μg	Streptomycin	10 μg
Lactobacillus	30 μg	Kanamycin	30 μg
Aztreonam	30 μg	Gentamycin	10 μg

medium plate, and the drug-sensitive test strips (Tianhe Microbial Reagent Co., Ltd., Hangzhou, China.) attached to the surface of the medium with a sterile forceps. Plates were placed in an incubator at 28˚C for 24 h, and the zones of clearing formed were compared with break-points from the Clinical Laboratory Standard Institute (CLSI) [14]. Characterization of strains as Susceptible (S), intermediate resistance (I) and resistant (R) was evaluated according to the criteria given in the Clinical Laboratory Standard Institute [14] and when not available, according to the antibiotic manufacturers' instructions.

2.4. Hemolysis, Protease and Lipase Activity

For the hemolytic activity, sheep blood agar plates were used to detect the activity of these factors. Bacteria strains were, inoculated onto Difco Bacto-agar (Difco Laboratories, Detroit, MI) with 4% Sheep Blood and incubated at 30˚C for 24 to 48 hr as previously described with some modifications [15] after which the presence or absence hemolytic activity was determined. After 18 - 24 hr of incubation at 37˚C, Protease activity was determined by a skim milk plate assay [16] with some modifications. Presence of a zone of protein casein hydrolysis was evidenced as protease activity. The lack of a transparent zone was a strong indication of no enzymatic activity.

Lipase activity was tested for by inoculating strains onto Spirit Blue Agar Plates containing an ester substrate. Strains were incubated at 37˚C for 24 to 48 hr and checked for areas of clearing around the colony growth [17]. Protease and Lipase production was scored as follows: −, no production; +, weak; ++, moderate; +++, high (**Table 3**).

Table 3. Results of virulent factors, AHLs production and Qse B/C genes in 15 isolates of *Aeromonas hydrophila*.

Strain	Protease	Hemolysis	Lipase	AHLs	QseB Gene	QseC Gene
ATCC 7966	+	*β*	++	+	+	+
JH-25	++	*α*	+	+	+	+
JH-26	++	*α*	+	+	+	+
JH-31	++	*α*	+	+	+	+
JH-33	++	*α*	+	+	+	+
JH-34	++	*α*	+	+	+	+
JH-36	++	*α*	+	+	+	+
JH-37	+++	*α*	+	+	+	+
JH-38	+++	*α*	+	+	+	+
JH-39	+++	*α*	+	+	+	+
DH-29	+	*α*	+	+	+	+
DH-41	+	*α*	+	+	+	+
JY-24	-	*β*	+	+	+	+
JY-25	-	*α*	+	+	+	+
TZ16	-	*β*	+	+	+	+

2.5. Biofilm Formation

All strains were grown in 3 ml of LB broth encased in polystyrene tubes at 37˚C for 24 h with shaking. Measurement of Biofilm formation was done according to the procedure described [18] with some modifications. After incubation, bacteria strains and medium were discarded and the tubes washed with distilled water and allowed to air dry before staining. Tubes were stained with 0.2% crystal violent solutions for 5 min, afterward, washed with distilled water and the dye eluted with ethanol. Absorbance was measured at OD_{570} nm. The experiments were repeated independently in three triplicates.

3. Results

3.1. Detection of AHLs, qseB/C Gene and Virulence Factors in *A. hydrophila*

C. violaceum CV026 have been reported to react well to Acetylated homoserine lactone (AHLs) having four to eight carbon acyl side chains yet ineffective to Acetylated homoserine lactone (AHLs) having acyl chains of C_{10} [19]. All strains demonstrated that they have the ability to produce AHL molecules (**Table 3**). A qseB/C gene was determined to be present if the amplicon of the expected size was pictured on the agarose gel. The results of PCR amplification demonstrated that all the tested strains produce qseB/C gene. The results of lipase, hemolysin and protease activity are summarized in **Table 3**. After 24 hours, all strains tested showed a zone of clearing around the bacterial growth, confirming lipolytic activity with ATCC 7966 showing the greatest lipolytic. This confirms that all the strains are capable of degrading lipase. Previous studies have revealed that Lipases are vital in invasiveness and establishment of infections [20]. The protease activity was measured by a zone of protein casein hydrolysis on a milk agar plate which was detected in all the strains after 24 hours. Three strains produced no protease activity (JY-24, JY-25, and TZ-16). Highest zone of clearing was exhibited by JH-37, JH-38, and JH-39 as compared to ATCC 7966 and DH-39 which had the least zone clearing. For the hemolysin activity, most strains displayed an Alpha hemolytic ring (α) on the blood agar plate, ATCC 7966, JY-24 and TZ-16 were the only strains which displaying a beta hemolytic ring (β).

3.2. Antibiotic Susceptibility

A total of 25 different kinds of drug-sensitive test strips representing 12 classes of antibiotics and their concentration per disk were used (**Table 2**). All isolates expressed some level of resistant to the class of antibiotics. 96.7% of the strains were Resistant to the β-Lactam.

There was a higher significant resistance to Tetracyclines. Among the Aminoglycosides tested, 100% susceptibility was recorded against streptomycin and gentamycin whereas kanamycin recorded 93.3% susceptibility. 60% of the strains showed intermediate resistance to Polypeptide. *Aeromonas* spp. have proven to be among the few microorganisms that have the ability to harbor different

chromosomal β-lactamase genes, as well as, *cphA*, *cepH,* and *ampH*, encoding classes B, C and D β-lactamases, respectively [21]. None of the isolates showed intermediate resistance to Aminoglycoside, Tetracycline, Sulfonamide, Fosfomycin, and monobactams (Figure 1). All isolates showed No Resistant to Aminoglycoside, Polypeptide, monobactam, and Carbapenems. The 15 isolated strains were not susceptible to the class Glycopeptide and β-Lactam.

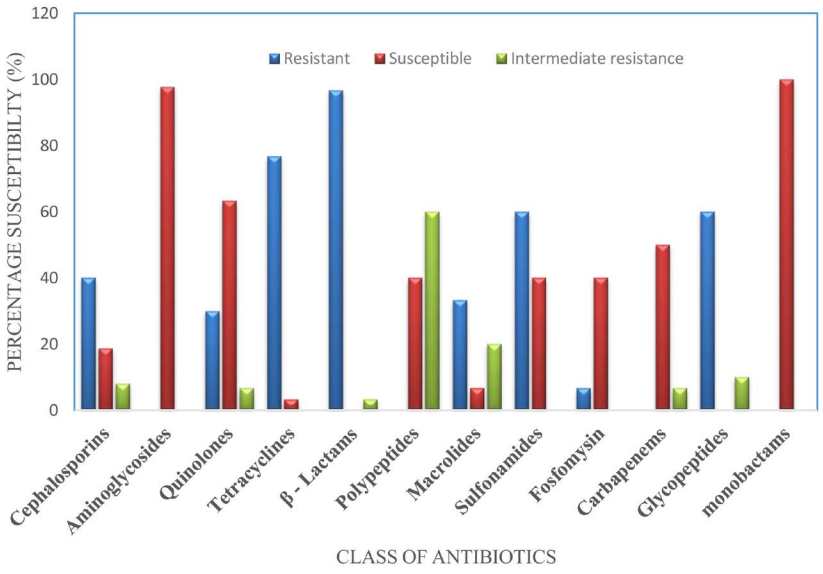

Figure 1. Percentage of Antibiotics Susceptibility levels of 15 isolated strains *of Aeromonas hydrophila.* Legend: Blue = Resistant, Red = Susceptible, Green = Intermediate Resistance.

3.3. Biofilm Formation

The biofilm formation in polystyrene tubes was observed after 24 h growth of *hydrophila* strains (Figure 2). JY-24 increased significantly in Biofilm formation when compared with the other tested strains, compared to JH-26 which had the lowest Biofilm formation after 24 h of incubation at 37˚C.

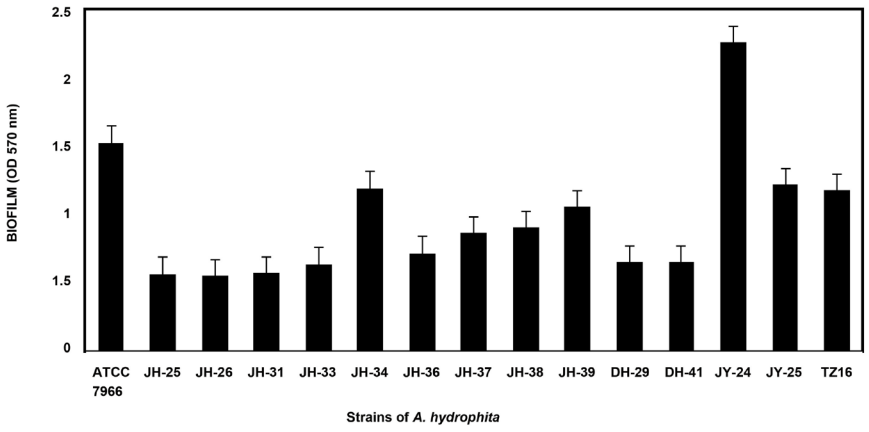

Figure 2. Biofilm formation of isolated strains compared to the Reference strain ATCC-7966. A total of 14 isolates were compared with the reference strain ATCC-7966.

4. Discussions

Over the years, *Aeromonas* species have been implicated as causes of numerous diseases like gastroenteritis, cellulitis either in humans or fish. Their multidrug resistance and pathogenic potentials could be a determining factor for this phenomenon. The type and gravity of disease are influenced by putative virulence factors possessed by this genus [17]. In the present study, we elucidated the producibility of AHLs, QseB/C genes detection, antibiotic susceptibility, virulence factors and biofilm formation of 15 *A. hydrophila* isolates. Invasiveness and toxin production contribute to the pathogenicity of bacteria [15] [22]. Previous studies have reported the importance of quorum sensing in *A. hydrophila* in terms of protease and virulence factor production. *A. hydrophila* regulates the production of protease and by using AhyR/C4-HSL-dependent QS system.

In this present study, we identified the virulence factors (hemolysin, protease, and lipase) that are associated with the pathogenicity of *A. hydrophila* and considered a threat to human health. It was observed that all clinical isolates in this study produced hemolysin and lipase. Only three of the isolates showed no phenomenon for protease production (**Table 1**). Red Blood plate hemolysis results showed that ATCC 7966, JY-24 and TZ-16 were beta-hemolytic and β-hemolysins have been identified as one of the important bacterial virulence factors, which are also responsible for outbreaks of diarrhea whiles the rest of the strains were alpha hemolytic. These results also suggest that these three bacterial isolates might be pathogenic to their host. A study by Al-Fatlawy and Al-Ammar (2013) showed that all isolates of *A. hydrophila* were positive for hemolysin production (β-hemolysin) [23] which agrees with the findings in our current study. β-hemolysin which is produced from *A. hydrophila* has a familiarity with the toxins produced in the cell producing enzyme, and a toxin called cytotoxic factors. Hemolytic toxins are evidence of pathogenic potential in aeromonads. Notwithstanding, non-hemolytic aeromonads have also been implicated as human pathogens. From our results, it was established that all the isolates were lipase producers. In a study by Pridgeon *et al.* (2013), it was reported that highly virulent isolates with hemolytic, protease and nuclease activity among the exotoxins such as hemolysis, aerolysin, and elastase and 5'-nucleotidase infected channel catfish [24]. The ability of *Aeromonas* species to produce virulence factors makes it a potential causative of diseases in fishes and humans [25]. Over the years, the most utilized antibiotic in both human and veterinary medicine is tetracycline, hence has developed the ability to counteract to innumerable aquatic bacteria [26] with unsusceptibility strains stretching from 7.7% to 11.5%. Individually the isolates were tested against 25 antibiotics and the inhibition zones after 24 hr was measured. Our results agree with previous studies where most strains of *A. hydrophila* displayed resistance to Ampicillin (100%) and cephalothin (86.7%) [27]. Ampicillin which belongs to the class of Penicillin recorded the highest resistance and this can be ascribed to β-lactamase agility in the resistant isolates which have been recorded in similar studies [28]. Resistance to

third-generation cephalosporin and imipenem is known to be associated with the derepression of the chromosomal enzymes. Resistance was also shown against isolates tested with tetracycline. 93.3% of the strain showed resistance to tetracycline and 60% showing resistance to doxycycline. The Resistance pattern against Tetracycline is similar to the findings of Goñi-Urriza *et al.* (2000), where Tetracycline showed resistance to *Aeromonas* species isolated from a river that receives wastewater discharge [29]. The existence of *tet* genes in the bacterial DNA is a contributing factor of tetracycline resistance [25]. The isolated strains showed significant susceptibility to the newer generation of fluoroquinolone (ciprofloxacin and norfloxacin). Fluoroquinolones have been reported by many researchers as a surest treatment for *Aeromonas* infectious diseases. 30% of the strains exhibited resistance to the quinolones. Gram-negative bacteria resistance to quinolones is partly influenced by the mutation in the quinolones resistance determining regions (QRDRs) which encompass the gyrA and parC gene and are subunits of the targeted enzymes of quinolones, DNA gyrase subunit A and topoisomerase IV, respectively [30]. These results are an indication that *A. hydrophila* has developed strong resistance to antibiotics and not effective for use in aquaculture, hence the need to develop alternative therapeutic agents to control them effectively.

Our result also corresponds to other works which showed that *A. hydrophila* produces quorum sensing signal molecules [31]. Bacterial biofilm formation is a complicated multifaceted mechanism, associated with attachment, quorum sensing, cell wall synthesis, metabolism, and stress response [32]. The increasing antimicrobial resistance and virulence of bacterial species are due to their ability to produce biofilms [7]. All the isolates from our study are strong biofilm producers. Igbinosa *et al.* (2015) revealed that almost 86% of isolated *Aeromonas hydrophila* produce biofilm [33]. Our results are therefore in agreement with their findings. This Characteristic of these isolated strains forming biofilm may lead to difficulty in management during infection and poses a serious health threat of public concern. *A hydrophila* has become an established opportunistic pathogen which largely survives in aquaculture environment and the gastro intestine of fish.

5. Conclusion

Our findings clearly demonstrated that there is a presence of QseB/C genes in *A. hydrophila* and also it produces some virulence factors. Additionally, *A. hydrophila* had the ability to produce AHLs molecules and it can be concluded from this study that Penicillin and tetracycline should be avoided for treating infections caused by *A. hydrophila.* Given the emerging antimicrobial resistance, antimicrobial susceptibility testing of isolates demonstrated in this study is essential to guide antibiotic selection. Moreover, fosfomycin could be an adequate treatment alternative in aquaculture. This study provides an understanding of bacterial pathogenesis and encourages new approaches of biological control

mechanisms for *A. hydrophila* infection and antibiotic resistance.

Acknowledgements

This work was financed by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank the anonymous reviewers for their constructive criticisms of the manuscript.

Authors Contribution

Conceptualization, W.C.; methodology and Investigation, W.C., E.K.S., and S.Z.; formal analysis, E.K.S.; data curation, E.K.S. and S.Z.; writing—Original draft preparation, E.K.S.; writing—Review and editing, W.C., and E.K.S.

Conflicts of Interest

The authors declare no conflict of interest.

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