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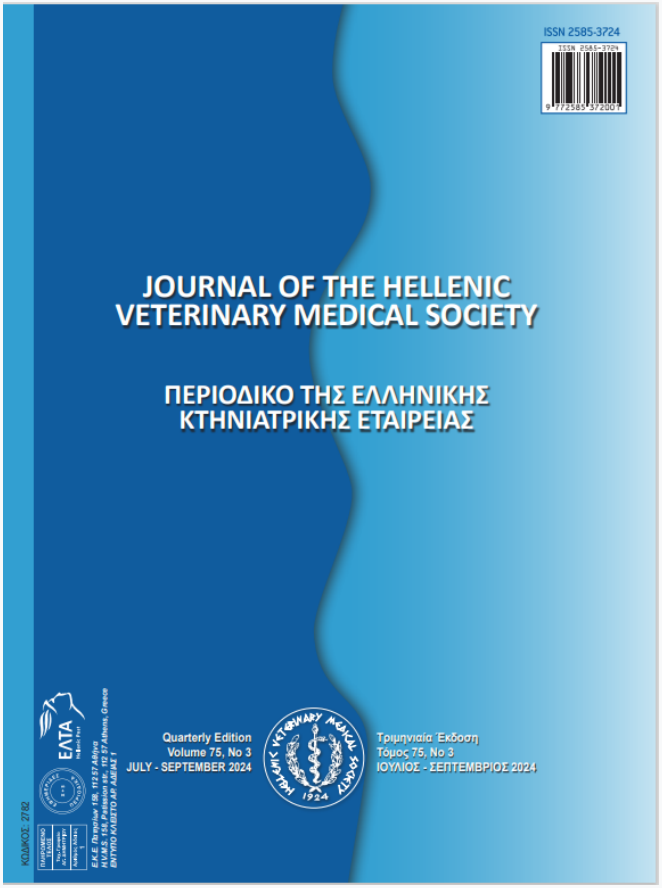

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Quorum Sensing System in Pathogenic *Aeromonas hydrophila*: Discovery of *LuxRI* Homologs *ahyI* and *ahyR* Genes Responsible for *N*-Acyl Homoserine Lactone Signal Molecules

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ABSTRACT: Pathogenesis intercellular communication system as called Quorum Sensing (QS) plays a crucial role in chemical communication between bacteria involving autoinducers, receptors and controls production of virulence factors in bacteria. *A. hydrophila* was reported to be regulated by QS pathways, indicating that it is involved in QS network regulation correlated with bacterial virulence. In *A. hydrophila* strains, it initiates disease process by preventing premature stimulation of immune system by critical gene expressions. *A. hydrophila* is fish pathogen which produces *N*-Acyl Homoserine Lactone (AHLs) QS signal molecules and possesses homologues of the model strain *Vibrio fischeri luxI* and *luxR* QS genes termed *ahyI* and *ahyR*, respectively. *A. hydrophila* secretes AHL signal molecule with *LuxI* and *ahyI* gene, and senses AHL signal molecule with *LuxR* and *ahyR* gene. In this work, 20 clinical *A. hydrophila* strains were analysed for presence of Quorum Sensing genes *ahyI* and *ahyR* by Polymerase Chain Reaction (PCR). The QS genes *ahyI* (78 bp) and *ahyR* (86 bp) were detected in a total of 20 strains. Thus, it has been proven that pathogen bacteria *A. hydrophila* possesses *LuxI/LuxR*-Type QS. This genomic analysis determined the comprehensive QS systems of *A. hydrophila*, which might provide novel treatment strategy regarding the mechanisms of virulence signatures correlated with QS. With identification of QS and all members of system (AHLs, AI, QSI e.g.), early diagnosis of disease is brought to agenda and it is expected to break new ground in diagnosis of fish diseases. As previously reported, these data add *A. hydrophila* to the world of bacteria now known to control gene expression through QS.

Keywords: Interbacterial communication, *A. hydrophila*, Quorum sensing system, *ahyI* and *ahyR* Genes, PCR

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INTRODUCTION

Quorum Sensing System (QS) that empowers bacteria to survive (Chu et al., 2015). With social behavior, bacteria communicate with each other through signal molecules they have produced, monitor whether they have reached certain majority, and trigger critical gene expressions as soon as they reach cell population. The "language" used for this intercellular communication is based on small, self-generated signal molecules called autoinducers. Thus, before bacteria reaches threshold value, it creates biofilm before immune system realizes bacteria and not stimulate host immune system prematurely, thus creating successful disease process (Saraçlı, 2006; Gupta and Kumar, 2022).

Elementary of communication between bacteria was with interpretation of "I think a bacterial community is stronger than few bacteria, and therefore they can overcome many obstacles", with first definition of "QS" QS indirectly (Smith, 1905). Currently, studies have shown that single-cell bacteria can communicate with each other and respond to changing environment. Nowadays, it is known that bacteria can synchronize subgroups of their genes and act together by using signal molecules.

Nowadays, bacteria have proven that using QS exhibit social act, monitor their numbers in environment and trigger critical gene expressions as soon as they realise that they have reached desired cell number (Chadha et al., 2022). QS regulates functions such as conjugation, secretion of virulence factors, antibiotics production, biofilm and bioluminescense (Miller and Bassler, 2001). Some phenotypes under control of QS system-dependent AHL, A. hydrophila produced biofilm and virulence factors by producing C_4-HSL and C_6-HSL molecules (Williams 2007).

Bacteria regulate collective action by sending signals through low molecular weight autoinducing molecules under controlling of QS. Clonal bacterial population performs hedging or division of labor due to its phenotypic heterogeneity. This explains pathogenicity power of bacteria socializing with *N*-Acyl Homoserine Lactone (AHLs) molecules under QS management (Strriednig and Hilbi, 2022). With QS, bacteria started to obtain resistance to antibiotics and caused decrease in success of antibiotic treatment (Saeki et al., 2022).

A. hydrophila is Gram-negative, rod-shaped, opportunistic, motile, non-spore forming bacteria (Austin and Austin, 2016). *A. hydrophila* is zoonosis bacteria. *A. hydrophila* cause Motile Aeromonas Septicemia (MAS) in fish (Nafiqoh et al., 2022) and result in serious mortality (Christy et al., 2019; Rahman et al, 2022). *A. hydrophila* is very toxic due to its structure. Scientists have proven that *A. hydrophila* is pathogenic bacteria (Seshadri et al., 2006). Secretion System (TTSS) invades virulence factors into host cell. Virulence secreted by pathogenic bacteria and translocated via TTSS are effector molecules responsible for inducing cell damage and apoptosis (programmed cell death). These virulences and toxins have been detected in pathogenic *A. hydrophila*, which causes disease in fish. All virulence factors are focused on destroying cell elements and killing cells (Seshadri et al., 2006).

QS is a hierarchical structure (Zhu and Winans 2001). It is accepted that *A. hydrophila* not asocial and uses complex intercellular communication systems to facilitate their adaptation to changing environmental conditions (Swift et al., 1994).

Gram$^-$ bacteria is synthesized and released by bacteria via *LuxI* chemical reaction products. *LuxR* chemical reaction product of another bacteria responds and binds to receptor protein and detects AHL molecule (e.g. *A. hydrophila*) (Raffa et al., 2005).

E. coli use *QseB* and *QseC* gene systems respond to bacterial communication and synthesize virulence factors. Inhibition of *QseC* interrupts crosstalk and significantly reduces virulence. Commonly AI molecules are AHLs molecules and this signaling mechanism has been proven by literatures. In *A. hydrophila*, there is communication with AHL molecules under management of QS, which is one of these AI molecules, as well as *QseB* and *QseC* systems in *A. hydrophila*. There is crosstalk between AHL and *QseB* and *QseC* gene systems in *A. hydrophila*. In *A. hydrophila*, autoinducers Al-1, Al-2 and AL-3 systems are responsible for biofilm in coordination with each other. *QseB* and *QseC* genes are also effective in regulating QS virulence factors of *A. hydrophila* (Kozlova et al., 2012; Sarkodie et al., 2019).

The aim of this research is to determine *ahyI* and *ahyR* genes responsible for release of AHL signal molecules belonging to QS that carry out interbacterial communication in *A. hydrophila* strains which pathogenic bacteria.

MATERIAL AND METHOD

Bacterial strains and growth conditions

A total of 20 *A. hydrophila* strains and *A. hydrophila* ATCC 7966 reference strain were investigated in this study (Table 1). In study, we used phenotypic identification assays all strains. Especially *ahyI* and *ahyR* sequencing to determine species identity of clinical *A. hydrophila* strains derived from sick fish suffering from MAS disease. Also, *A. hydrophila* ATCC 7966 was considered as a reference strain. The stock cultures were prepared in TSA supplemented with 20% sterile glycerol and stored at −80°C. Prior to each experiment, bacteria were grown in TSA at 25°C (Ausubel et al., 1988). *A. hydrophila* was grown on Tryptic Soy Agar (TSA), Aeromonas Isolation Base Agar (AIBA), Tryptic Soy Broth (TSB) and Glutamate Starch Phenol Red (*Pseudomonas Aeromonas* Selective Agar Base acc. to KIELWEIN, GSP). Inoculate all media (TSA, AIBA, TSB, GSP) with test organisms and incubate at 25°C for 24 h aerobically.

Identification assays

Identification of *A. hydrophila* strains was confirmed by making yellow colonies on GSP, Gram staining assay, vibriostat assay, O/F assay, strains motility assay, catalase assay, cytochrome oxidase assay (Cappuccino and Sherman, 1992).

PCR Reaction

Colony PCR method was applied to detect *ahyI* and *ahyR* genes in clinical *A. hydrophila* bacterial samples, and *A. hydrophila* ATCC 7966 strain was used as a positive control. For this purpose, 2-3 colonies were taken from the overnight agar culture of the bacterial samples with a sterile toothpick and 500 µL of sterile pure water was added in a 1.5 mL sterile Eppendorf tube, the colonies were suspended in pure water and kept in the heat block for 5 min. Eppendorfs were kept to cool at room temperature and then centrifuged at 14000 rpm for 5 minutes, and the supernatant formed after centrifugation was used in PCR studies. For this study, the presence of *ahyI* and

Table 1. Phenotypic identification schemes in *A. hydrophila* strains

Strains	Isolation	Gram stain	Motility	Cytochrome oxidase	O/F	Catalase	0/129 Vibriostat	AIAB (Coloni pigment)	GSP Agar (Coloni pigment)	β-Hemolysis Blood Agar
A. hydrophila ATCC 7966	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH RSKK 05049	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH SAHA	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH S	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH J	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 2	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 3	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 4	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 12.1	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 14	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 15	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 16	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 108	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 113	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 216	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 217	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 219	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 220	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 222	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 230	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)
A. hydrophila AH 232	Liver-Kidney	-	+	+	+/+	+	R	Yellow-green	Yellow	+ (β)

R: Resistance

ahyR gene regions, approximately 78 and 86 bp were investigated in the strains by PCR and four pairs of primers suitable for the gene region to be investigated were used and the reaction volume was carried out in a total of 25 µl *(ahyI* gene; Forward primer CTTTCGCAATCGCGTCTTCT; Reverse primer ATCGAAACTGTCCTGCTCCA; *ahyR* gene; Forward primer CCCATCCTCTCCTGGATGTC, Reverse primer CTCCTGAGGGTCATCTTCCC). PCR reaction; It was carried out as 1 cycle of 5 minutes at 95°C, 30 minutes at 95°C, 30 minutes at 50-65°C, 1.30 minutes at 72°C and 10 minutes at 72°C (Azevedo et al., 2017).

Agarose Gel Imaging: The products resulting from PCR were mixed with 2% agarose gel containing 0.5 µg/ml ethidium bromide was run in TAE buffer at 70V with 50 bp DNA marker in electrophoresis device (Sentromer DNA, 2020; Fernández vd., 2008). The reaction product without DNA was used as a negative control and the results were evaluated in comparison with the *A. hydrophila* ATCC 7966 strain.

RESULTS

A. hydrophila Strains

Biochemical properties of *A. hydrophila* strains obtained from different collections isolated from different ailment fish were studied. According to these results, it was determined that all of strains were resistant to Gram$^-$, cytochrome oxidase and catalase (+), vibriostat, fermentative in O/F test and motile in motility assay. Thus, all *A. hydrophila* strains studied were confirmed.

Identification of *ahyI* and *ahyR* Genes

AHL-dependent QS system based on *ahyRI* locus, presence of *ahyI* gene and *ahyR* gene was established by PCR in *A. hydrophila*. DNA extraction was conduct from strains and concentration and purity values were measured in UV spectrophotometer. Primers suitable for target genes were synthesized and amplicon lengths were determined as 78 bp for *ahyI* gene and 86 bp for *ahyR* gene (Table 2). *ahyI* and *ahyR* genes depend on QS system management was determined in all *A. hydrophila* strains (Figure 1., Figure 2.).

Table 2. *ahyI* and *ahyR* genes and UV-Spectrophotometer results of the strains

Strains	*ahyI* gene DNA Sequence (5' - 3')	Target Gene	*ahyI* gene (78 bp amplicons)	Strains	*ahyR* gene DNA Sequence (5' - 3'	Target Gene	*ahyR* gene (86 bp amplicons)	ng/ul	260/280	260/230
ATCC 7966		*ahyI*	+	ATCC 7966		*ahyR*	+	717.10	2.06	1.45
AH RSKK 05049	FP: CTTTCGCAATCGCGTCTTCT RP: ATCGAAACTGTCCTGCTCCA	*ahyI*	+	AH RSKK 05049	FP: CCCATCCTCTCCTGGATGTC RP: CTCCTGAGGGTCATCTTCCC	*ahyR*	+	75.16	2.14	1.79
AH SAHA		*ahyI*	+	AH SAHA		*ahyR*	+	288.01	2.00	1.86
AH S		*ahyI*	+	AH S		*ahyR*	+	585.19	2.05	1.91
AH J		*ahyI*	+	AH J		*ahyR*	+	435.69	2.03	2.04
AH 2		*ahyI*	+	AH 2		*ahyR*	+	96.12	1.93	1.11
AH 3		*ahyI*	+	AH 3		*ahyR*	+	1346.67	1.58	1.11
AH 4		*ahyI*	+	AH 4		*ahyR*	+	84.42	2.02	1.34
AH 12.1		*ahyI*	+	AH 12.1		*ahyR*	+	85.95	2.06	2.10
AH 14		*ahyI*	+	AH 14		*ahyR*	+	137.54	2.08	1.93
AH 15		*ahyI*	+	AH 15		*ahyR*	+	167.82	1.82	1.06
AH 16		*ahyI*	+	AH 16		*ahyR*	+	302.90	2.06	1.95
AH 108		*ahyI*	+	AH 108		*ahyR*	+	440.81	2.06	1.77
AH 113		*ahyI*	+	AH 113		*ahyR*	+	553.01	2.13	1.92
AH 216		*ahyI*	+	AH 216		*ahyR*	+	365.10	2.01	1.73
AH 217		*ahyI*	+	AH 217		*ahyR*	+	145.31	1.72	1.01
AH 219		*ahyI*	+	AH 219		*ahyR*	+	818.60	2.27	2.17
AH 220		*ahyI*	+	AH 220		*ahyR*	+	553.60	1.99	2.01
AH 222		*ahyI*	+	AH 222		*ahyR*	+	365.41	1.93	1.78
AH 230		*ahyI*	+	AH 230		*ahyR*	+	251.45	2.16	1.88
AH 232		*ahyI*	+	AH 232		*ahyR*	+	342.86	1.98	1.99

FP: Forward Primer
RP: Reverse Primer

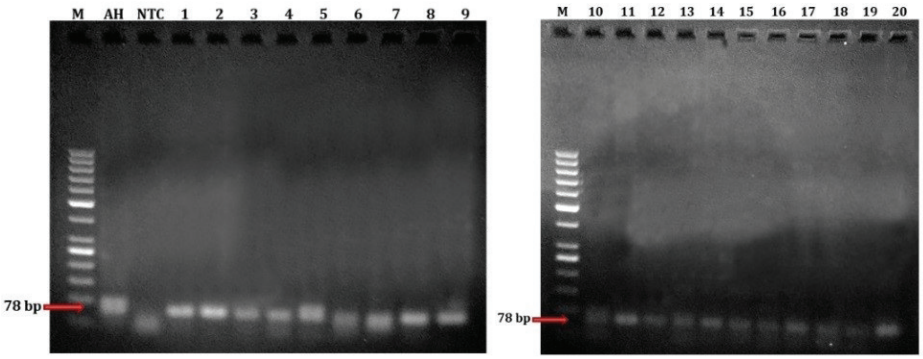

Figure 1. Clinical strains analyzed by PCR for QS-controlling *ahyI* genes *A. hydrophila* identifications. Lane M 50-bp DNA marker, lane AH PTC reference strain (ATCC) 78-bp, lane NTC NTC, lane 1 *A. hydrophila* strain, and lane 1-20 targeting *ahyI* genes *A. hydrophila* strains (PTC: Positive control, NTC: Negative control)

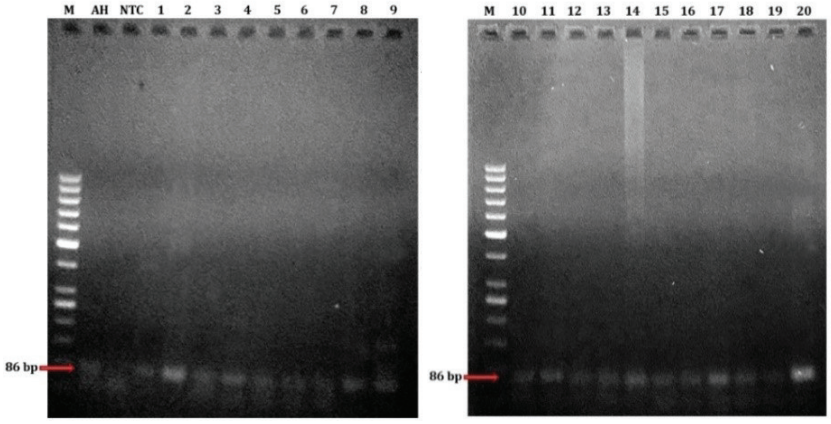

Figure 2. Clinical strains analyzed by PCR for QS-controlling *ahyR* genes *A. hydrophila* identifications. Lane M 50-bp DNA marker, lane AH PTC reference strain (ATCC) 86-bp, lane NTC NTC, lane 1 *A. hydrophila* strain, and lane 1-20 targeting *ahyR* genes *A. hydrophila* strains (PTC: Positive control, NTC: Negative control)

DISCUSSION AND CONCLUSION

Researchers have long analyzed unicellular/monoclonal organisms in clinical bacteriology and microbiology research, adhering to principle of "pure strains". While this principle is very true, scientists are now increasingly uncovering socio-microbiological aspects of bacteria to understand bacteria, also with research questions such as multicellular/polyclonal lifestyle, biofilm, and QS.

Similarities of signals used by bacteria with artificial neural networks attracted attention of scientists, and based on finding that bacteria contain neural networks, idea of a low-level intelligence structure for bacteria was also strengthened (Hellingwerf, 2005).

Antimicrobials such as probiotics (Austin and Sharifuzzaman, 2022), immunostimulants (Zhang et al., 2022) and vaccines (Tripathi and Dhamotharan, 2022), which are alternative strategies for good management of fish diseases, have been used successfully. However, there is need for new alternative methods to these methods, and QS is one of them (Defoirdt et al., 2004; Bruhn et al., 2005).

QS is the regulation of gene expression depending on the cell population density. A variety of bacterial virulence traits, including secretion of extracellular enzymes, biofilm, bacterial motility as well as bacterial secretion systems, have been reported to be regulated by QS systems (Tanhay Mangoudehi et al., 2020).

In onslaught of bacterial pathogenicity, because of QS is complex, signaling molecules produced by bacteria are biomarkers for diagnosis and follow-up of bacterial infections. Detection of QS signal molecules may be more useful at very early stage as tool to detect bacterial infections (Boyen et al., 2009). There are currently no comprehensive reports identifying AHL molecules, communication system of important

bacterial pathogens.

For this reason, bacterial resistance and grounds on which resistance develops have become a common threat to humanity in recent years. As a result of discussion over time of Robert Koch's assumptions published in 1884, Bill Costerton warned in 1978 that there were resistant bacteria. At this point, when concept of "communication between bacteria", which Smith (1905) brought to agenda for first time, began to be considered together with concept of "QS", new dimension was passed in evaluation of infectious diseases (Bayrakal and Baskın, 2018).

It was reported by Kirke et al. (2004) and Swift et al. (1997) that by PCR, encoded production of *N*-butanoyl-$_L$-homoserine lactone (BHL) signal molecule, which is AHL molecule for QS in *A. hydrophila*, with *ahyI* and *ahyR* genes which *A. hydrophila*'s virulence (biofilm and serine protease). In the presented study, it was observed that both *ahyI* and *ahyR* genes of all *A. hydrophila* strains were determined by PCR method.

It was reported by Swift et al. (1999) and Lynch et al. (2002) that the *A. hydrophila* produced virulence with *ahyR* gene BHL molecule under management of QS. Similarly, *ahyR* gene was detected in all *A. hydrophila* strains in our study.

Swift et al. (1999) stated that *ahyI* gene encodes the protein required for synthesis of *N*-butanoyl-$_L$-homoserine lactone (C_4-HSL) QS signal molecule in *A. hydrophila* and that molecules can be prevented by inactivating *ahyI* gene in *A. hydrophila*. Similary in this study, both *ahyI* and *ahyR* genes responsible for virulence factors were detected in *A. hydrophila* strains.

There is interspecies communication between (crosstalk) AHL in *A. hydrophila* and *E. coli QseB* and *QseC* gene system (Kozlova et al., 2012; Sarkodie et al., 2019). *E. coli* bacteria use *QseB* and *QseC* gene systems to respond to bacterial communication and to synthesize virulence factors. Inhibition of *QseC* interrupts crosstalk and significantly reduces virulence.

It was reported by Sarkodie et al. (2019) that *E. coli* bacteria respond to bacterial communication with *QseB* and *QseC* gene systems and use it to synthesize virulence factors. In same study, it was reported that inhibition of *QseC* interrupted intraspecies communication and significantly reduced virulence. It was reported by Sarkodie et al. (2019) that the *A. hydrophila* communicated with AHL molecules, virulence factors production, and in similarly it was reported by Kozlova et al. (2012) that *A. hydrophila* also has *QseB* and *QseC* systems, as well as communication with AHL molecules under of QS management. Interspecies communication between AHL and *QseB* and *QseC* gene systems in *A. hydrophila* has been reported. While it is known that *A. hydrophila* communicates intraspecifically with QS, *A. hydrophila* draws attention with emphasis in this study when it communicates between species. Inhibition of QS is very important in treatment world, as this clinical fish pathogen is organized with QS, and causes various diseases in fish with its virulence factors by acting simultaneously.

It was reported by Smith (1905) that many bacteria are stronger than a few bacteria, but that these few bacteria can overcome obstacles together. Years after these words, it has been proven that bacteria communicate with each other and can respond (Baskın, 2005). With disruption of communication system between bacteria, bacteria will not be able to act in coordination, they will not be able to colonize host, and as a result, they will not be able to show successful disease process (Rasko and Sperandio, 2010).

Researchers have stated that billions of bacteria collectively carry out same command in their experiment by putting bacteria into communication with each other with program loaded in their DNA. Scientists state that billions of bacteria that can communicate with each other can be managed at the same time and directed to certain tasks. They point out that in future, smart biological devices will also be reflected in daily life. They stated that smart biological devices can be successfully used in tissue regeneration, especially in medicine (Aleksandra, 2010).

In order to cause disease in fish, bacterial pathogens trigger critical gene expressions such as synthesis of virulence and show their effects on the host as soon as they sense each other with signal molecules and realize that they have reached desired majority. One of answers to question of how bacteria, known as one of simplest creatures, cause serious losses in natural environment and aquaculture units by causing disease in fish consisting of billions of systemized cells: QS. In fact, biofilm (Filik, 2019), which is the most effective virulence factor in QS-controlling; It has been described as the "city of microbes" (Watnick and Kolter, 2000), which is formed by gathering of many bacteria attached to an inanimate or living surface in mucous structure secreted. All this proves how smart they are. QS is indicator of when bacteria

act by thinking. Cessation of bacterial communication between them will largely end its negative effects on fish (Nurcan, 2010; Filik, 2020).

Random use of antibiotics in drug treatment of bacterial diseases causes development of resistance. Thus, we have faced a post-antibiotic era in which our ability to fight bacteria has diminished and need for new strategies to deal with disease has increased. Discovery that bacteria regulate their virulence factors using the interbacterial communication system has made blocking interbacterial communication system an attractive target for treatment (Fan et al., 2022).

Important to focus on strategies to prevent, cleave or inhibit production of QS molecules, and to prevent acquisition of QS signal. In this sense, cutting off communication in bacterial world is an alternative method of disease prevention in aquaculture. Studies to obtain antibacterial effects by preventing communication between bacterial cells are seen as a promising field for the future. Thus, further work will be required to refine our understanding of the multiple regulatory inputs which impact on *ahyI* expression and hence quorum sensing in *A. hydrophila*.

As a result, *A. hydrophila* secretes AHL signal molecule with *LuxI* and *ahyI* gene, and detects AHL signal molecule with *LuxR* and *ahyR* gene. In this study, both *ahyI* and *ahyR* genes responsible for pathogenicity and virulence in management of QS system were detected in all *A. hydrophila* strains by PCR analysis using 20 bp long primers.

As the list of bacteria that employ QS systems quaite wide. Because many crucial pathogens use QS to regulate virulence, strategies designed to interfere with these signaling systems will likely have broad applicability for control of disease-causing bacteria. In the future, it will be intriguing to see whether pathogens utilize QS of their pathogenic lifestyle and, if so, whether production of the signal molecules, AHL or otherwise, can be exploited to control ailments.

Thus, it has been proven that pathogen bacteria *A. hydrophila* possesses the *LuxI/LuxR*-Type QS. When all these situations are evaluated, it is very important to stop QS and fight while bacteria have not communicated with each other and have not yet formed disease. Detection and destruction of signal molecules during communication of pathogens will draw successful profile in prevention of disease formation. In additional, detection of QS signal molecules and stopping formation of these molecules brings up concept of early diagnosis in disease, and in this case, it is aimed to break new ground in prophylaxis.

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CONFLICT OF INTEREST

Authors have no conflict of interest to declare for the publication of the present work

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