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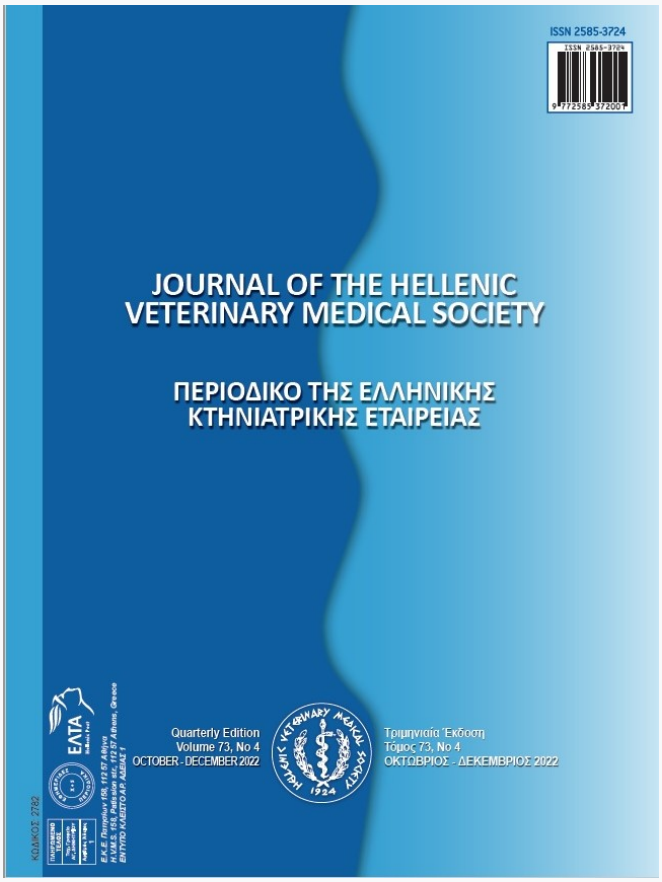

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Detection of Quorum Sensing System (Cell to Cell Communication) Using Marker Strains in *Vibrio alginolyticus* Strains and Determine Virulence under Master of this System

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ABSTRACT: There are intercellular communication and social behaviors, including Quorum Sensing System, QS, (cell to cell communication) in bacteria. Aim of present study was to investigate production of QS signal molecules (*N*-acyl homoserine lactones, AHLs) and also determine virulence factors regulation of QS in *V. alginolyticus*. QS of *V. alginolyticus* was proved by cross validation assay via AHL using marker bacteria strains (*Agrobacterium tumefaciens* NT1 and *Chromobacterium violaceum* CV026). *V. alginolyticus* did not produced *N*-butanoyl homoserine lactone (BHL), while *N*-(3-okzododekanoyl)-L-homoserine lactone (OdDHL) production was detected in *V. alginolyticus*. The presence of virulence factors, such as biofilm, pigment, rhamnolipid, hemolysis, elastase, protease and amylase production investigated in *V. alginolyticus* as phenotypically.*V. alginolyticus* was found produce biofilm, pigment, amylase and elastase; however, it was not determined rhamnolipid, hemolysis and protease virulence.

Keywords: Quorum sensing; signal molecules; *N*-acyl homoserine lactones (AHLs); *Vibrio alginolyticus*; virulence factors

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INTRODUCTION

Quorum Sensing System (QS), which is cell-to-cell communication between bacteria, has been shown to regulate virulence factor production in different aquatic pathogens (Natrah et al., 2011). Sheng et al., 2012 demonstrated that the expression of the type VI secretion system (T6SS) in *Vibrio alginolyticus* was under the regulation of QS and alternative sigma factor.*V. alginolyticus*, Gram-negative bacteria, was commonly found in temperate marine ecosystems (Reilly et al., 2011; Gong et al., 2020). *V. alginolyticus* is an important fish pathogen that is isolated as a causative agent of Vibriosis in cultured gilthead sea bream *Sparus aurata* L. and sea bass *Dicentrarchus labrax* L. in Mediterranean coastal areas like Greece, Spain, and grouper, large yellow croaker, kuruma prawn, abalone and carpet shell clam throughout the world (Aguirre-Guzman et al., 2004). *V. alginolyticus* is not only a fish pathogen, but also a pathogen of aquatic invertebrates, mainly bivalves (Lattos et al., 2021). Thus, *V. alginolyticus* represents a severe pathogen that raises public concerns and causes huge economic loss in aquaculture (Gong et al., 2020).

Some behaviors are seemingly futile when performed by a single bacterium acting alone. However, we know that bacteria perform these tasks in an effective way. How are bacteria capable to do so? We now understand that, through the process called QS, bacteria synchronously control the gene expression response, which is managed via cell density, cross talk, building infrastructure for the disease, virulence and species complexity. QS allows bacteria to switch between two distinct gene expression programs. One that is favored at low cell density for individual, asocial behaviors, and the other is favored at a high cell density for social, group behaviors (Ng and Bassler, 2009).

Intercellular communication is based on small, self-generated signal molecules, which are called autoinducers. *QS* affords pathogenic bacteria mechanisms to minimize host immune system responses by delaying the production of tissue-damaging virulence until sufficient bacteria have amassed and prepared to overwhelm host defense mechanisms and establish infection (Deep et al., 2011). In pursuit of finding new drugs and new drug targets, it has been realized that bacteria express their pathogenicity in a cell density-dependent manner known as QS (Kalia et al., 2011).

Bacteria that use QS communication circuits regulate a diverse array of physiological activities. These processes include virulence, biofilm, plasmid conjugation, antibiotic production, motility and sporulation (Eickhoff and Bassler, 2018; Jiang et al., 2019). Gram-negative bacteria use *N*-acyl homoserine lactones (AHLs) as autoinducers, and Gram-positive bacteria use processed oligo-peptides to communicate. Recent advances indicate that cell-cell communication via autoinducers occurs both within and among bacterial species (cross talk) (Miller and Bassler, 2001). *V. alginolyticus* bacteria communicated with other bacteria through QS. Emergence of multi-drug resistant *V. alginolyticus* and the prohibition of antibiotics both require the development of new anti-virulence strategies and therapeutic agents with alternative modes of action (Liu et al., 2020a).

AHLs play a crucial role in various infection-related biological processes of marine Vibrio species, including survival, colonization, invasion, and pathogenesis. With the increasing problem of antibiotic abuse and subsequently the emergence of drug-resistant bacteria, studies on AHLs are therefore expected to bring potential new breakthroughs for the prevention and treatment of *V. alginolyticus* infections.

There are very few studies on QS of *V. alginolyticus*. The study is among first studies in literature that aimed to detect virulence factors under management of QS and QS of *V. alginolyticus*. A total of three starins of *V. alginolyticus* were collected and individually screened for QS activity using a marker (reporter) strain, *C. violaceum* and *A. tumefaciens* by plate assay. Aim of this study was to determine whether *V. alginolyticus* has QS responsible for disease causing, bacterial communication system, via mutant *C. violaceum* CV026 or mutant *A. tumefaciens* NT1 marker strains. It was also aimed to investigate biofilm, pigment, rhamnolipid, elastase, protease, amylase and hemolysis under the regulation of QS in *V. alginolyticus*.

MATERIAL AND METHODS

Bacterial strains and culture conditions

V. alginolyticus (3 strains) was used in study. The three *V. alginolyticus* strains were isolated from the anterior kidneys of diseased cultured sea bass. The pathogen was produced at 25°C in Tryptic Soy Agar (TSA) and *Pseudomonas aeruginosa* PAO1 (positive control) was produced at 30°C in Luria Bertani (LB) and was kept at -80°C and 20% glycerol. AHL signal molecules *C. violaceum* CV026 (McClean et al.,

1997) and *A. tumefaciens* NT1 were detected (Ravn et al., 2001). Strains solidified containing 1.2% agar and gentamicin (20 μg/ml) for *A. tumefaciens* NT1; kanamycin (20 μg/ml) for *C. violeceum* CV026 was produced in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) medium.

Methods

Detection of AHLs

According to the principle, a color change colony reveals the presence of AHL molecules (Bruhn et al., 2005). As positive control of *P. aeruginosa*, the presence of OdDHL signal molecules in *V. alginolyticus*. Detection of AHLs was achieved using either the *C. violaceum* CV026 strain bioassay (McClean et al., 1997) or the *A. tumefaciens* NT1 strain bioassay (Ravn et al., 2001).*C. violaceum* CV026 (a mini-Tn5 mutant) was used as an indicator strain for the detection of C4-HSL. *C. violaceum* CV026 responds AHLs by inducing the synthesis of the purple pigment violacein. *A. tumefaciens* NT1, carrying the plasmid pZLR4, was used to detect C12-HSL. *A. tumefaciens* NT1 produces a green color in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) in response to the AHLs with N-acyl chain length from six to 12 carbons. These strains were grown in or on LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) solidified with 1.2% agar when required and supplemented with appropriate antibiotics (*A. tumefaciens* NT1 Gentamycin 20 μg/ml; *C. violaceum* Kanamycin 20 μg/ml).

Screening for BHL in agar

V. alginolyticus was grown on commonly used laboratory media Tryptic Soy Agar (TSA). Inoculate plates (TSA) with test organisms and incubate at 25°C for 24 h aerobically. *V. alginolyticus* and *C. violaceum* CV026 grown on commonly used laboratory special media LBA for AHLs (BHL) detection reciprocatively. Inoculate plates (LBA) with test organisms and incubate at 30°C for 24 h aerobically. A marker strain, *C. violaceum* CV026 (mini-Tn5 mutant of wild type strain), deficient in autoinducer synthase which requires exogenous addition of BHL to produce violacein (purple) was developed for studying various QS mechanisms, and it offers a convenient tool for the biological assay. This system provides extremely sensitive detection of AHLs. BHLsignal molecule of QS was determined with purple color formation (McClean et al., 1997).

Screening for OdDHL in agar

V. alginolyticus was grown on commonly used laboratory media TSA. Inoculate plates (TSA) with test organisms and incubate at 25°C for 24 h aerobically. *V. alginolyticus* and *A. tumefaciens NT1* grown on commonly used laboratory special media LBA for AHLs (OdDHL) detection reciprocatively. Inoculate plates (20 μg X-Gal added LBA) with test organisms and incubate at 30°C for 24 h aerobically. The marker strain *A. tumefaciens* NT1 lacks the Ti plasmid and contains two plasmids, that encode the *traR* and *traI-lacZ* fusion genes. This system provides extremely sensitive detection of AHLs. It stimulates OdDHL molecule of *A. tumefaciens* NT1 strain by effect of X-Gal substance in medium. *A. tumefaciens* NT1 changes color upon stimulation of the OdDHL molecule. OdDHL signal molecule of QS was determined with green color formation (Ravn et al., 2001).

Cross-validation test for AHL molecules

AHL production was examined with marker strains *C. violaceum* CV026 and *A. tumefaciens* NT1. The results were compared with *P. aeruginosa* PAO1.

Biofilm assay

Biofilm assay was carried out with minor modifications using the previously described protocol of O'Toole and Kolter, 1998. Briefly, *V. alginolyticus* strains were grown in 1% NaCl LBS for 16 h, normalized to identical density based on OD_{600} and then diluted 1:100 into fresh LBS medium. Diluted culture (100 μL) was pipetted into sterile 96-well flat-bottomed tissue culture plates and incubated at 25°C for 24 h. Following incubation, the wells were rinsed with distilled water and the remaining biofilm-associated cells were stained with 1% crystal violet for 15 min. The excess stain was then washed off gently by slowly running water and the biofilm attached to the 96-well plates was photographed. To quantify biofilm, stained biofilms were solubilized with 2×200 μL of 95% ethanol. The ethanol volume was brought to 4 mL with dH_2O and absorbance determined at 490 nm in a spectrophotometer (Ye et al., 2008) (Note, OD: Optical Density).

Pigment assay

V. alginolyticus strains were grown overnight at 25°C in LBS containing 1% NaCl. Bacterial cell cultures were then diluted to an OD_{600} of 0.05 in either TSB or TSB containing 5 mM $_{L}$-tyrosine and incubated at 25°C with shaking. At various time points, 1-ml

samples were taken from the culture, and the supernatants were collected by centrifugation (12,000 x g, 2 min). Pigment production was estimated in the supernatant by measuring the absorbance at 405 nm. Cell densities were determined by counting CFU on Tryptic Soy Agar or by measuring the OD_{600} (Croxatto et al.,2002).

Rhamnolipid assay

The test carried out using agar M9-glutamate minimal medium agar plates; containing 0.2 g of cetyltrimethylammonium bromide (CTAB) and 5 mg/l^{-1} methylene blue. *V. alginolyticus* strains were grown in the medium at 25°C for 24 hours. 20 µl of overnight LBB cultures of these strains were taken and dropped into the middle of M9-glutamate minimal medium agar plates and incubated at 25ºC, after incubation, the clear zone around the bacterial colony was accepted as an indicator of rhamnolipid activity. Sterile LBB was used as a negative control. The results were evaluated by comparing them with the *P. aeruginosa* PAO1 strain. The ramnolipid production test was made three parallels (Siegmund and Wagner, 1991).

Elastase assay

The elastase activity of *V. alginolyticus* strains was determined using Elastin-Congo red (ECR, Sigma-Aldrich). Briefly, *V. alginolyticus* was grown in LBBS with 1% NaCl at 25°C for 14 h. The supernatant was obtained by at 12,000 rpm at 4°C for 2 min. A hundred microliters of supernatant was added to 900 µl of buffer (100 mM Tris, 1 mM $CaCl_2$, pH: 7.5, containing 20 mg of ECR). The mixture was incubated at 25°C for 3 h by shaking at 200 rpm. Supernatant without indissoluble ECR was measured at 630 nm. (Ohman et al., 1980; Jian et al., 2019).

Protease assay

V. alginolyticus strains were grown on Tryptic Soy Agar (TSA) containing 1% NaCl and 2% skimmed milk for 24 hours at 25°C after that the clearing zones around the colonies were observed (Dong et al., 2005).

Amylase assay

V. alginolyticus were grown on TSA plates containing 1% NaCl and 2% starch for 24 hours at 25°C and the clearing zones around the colonies were observed (Swift et al., 1999).

Haemolysis assay

V. alginolyticus were grown on TSA plates containing 1% NaCl and 5% human blood for 24 hours at 25°C and the clearing zones around the colonies were observed (Swift et al., 1999).

RESULTS

Detection of AHLs by marker strains in *V. alginolyticus*

According to the principle, a color change colony reveals presence of AHL molecules (Bruhn et al., 2005). *P. aeruginosa* (positive control strain), *V. alginolyticus* (test strain), *A. tumefaciens* NT1 and *C. violaceum* CV026 (marker strains of QS system) are shown in figure 1. It was determined that were no BHL signal molecules in *V. alginolyticus* since *C.violaceum* CV026 marker strain did not change from cream to purple color. Hovewer, *A.tumefaciens* marker strain changed from cream to green color. So, *V. alginolyticus* produced OdDHL while it did not produce BHL. As a result, *V. alginolyticus* were proved to be formed disease using OdDHL belong to QS bacterial communication (Figure 2). X-Gal added to agar for detection of OdDHL signal molecule.

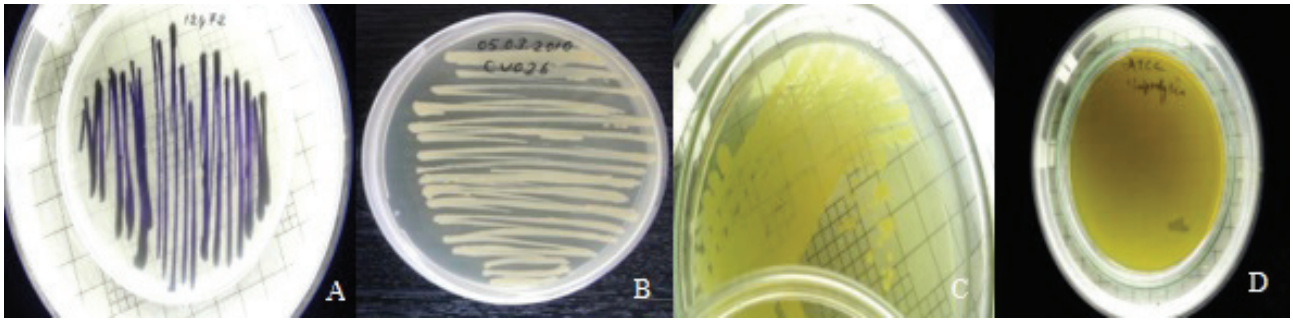

Figure 1. A. Original strain *C. violaceum* 12472 (purple color), B. QS system marker strain *C. violaceum* CV026 (cream color), C. QS system marker strain *A. tumefaciens* NT1 (cream color), D. *V. alginolyticus* (cream color), respectively

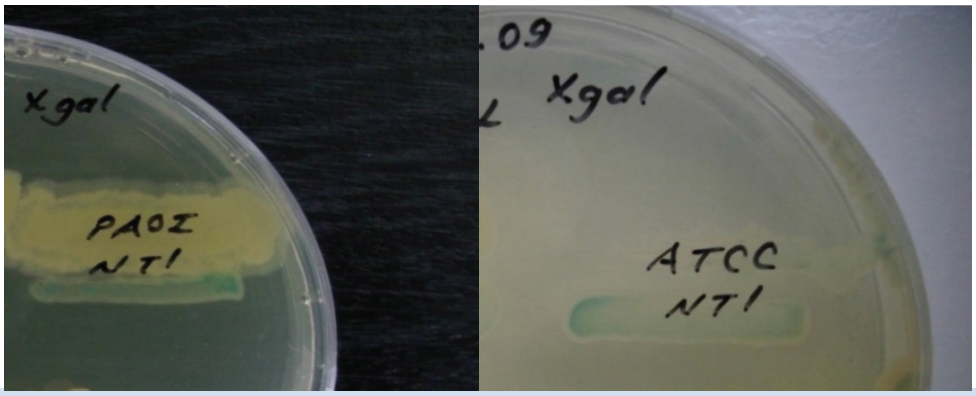

Figure 2. Agar plate assays for screening for production of AHL in *V. alginolyticus* using marker strain. Marker strains were streaked parallel to the tested bacteria. QS in *V. alginolyticus*: Detection of AHL by *A. tumefaciens* NT1. Green pigmentation was visible after 24 h of incubation as the AHLs produced by the *V. alginolyticus* diffused through the agar and activated the marker strain. *P. aeruginosa* PAO1 were included as positive

Biofilm results

V. alginolyticus was found produce biofilm (Figure 3). Finally, biofilm formation was measured by determining the absorbance at 490 nm wavelength using a spectrophotometer (Table 1).

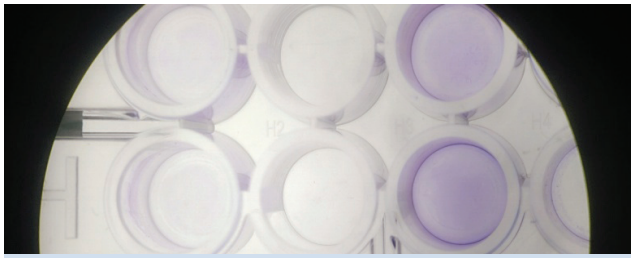

Figure 3. Biofilm formation in *V. alginolyticus* at third well (first and second well negative control, respectively)

Table 1. Optical density measurements of biofilm in strain at a wavelength of 490 nm for *V. alginolyticus*

***V. alginolyticus* ATCC**	***V. alginolyticus* V5**	***V. alginolyticus* V6**	***P. aeruginosa* PAO1 (positive control)**
0.099	0.141	0.138	0.070
0.093	0.172	0.091	0.068
0.093	0.076	0.074	0.080
0.093	0.173	0.069	0.071
0.084	0.128	0.073	0.078
0.063	0.084	0.071	0.073
0.085	0.097	0.080	0.086
0.098	0.116	0.101	0.121
0.095	0.185	0.113	0.069
0.094	0.161	0.221	0.069
0.089	0.191	0.074	0.069
0.089	0.094	0.092	0.077
0.084	0.161	0.078	0.065
0.094	0.069	0.079	0.071
0.098	0.178	0.086	0.071
0.075	0.094	0.117	0.103
0.099	0.186	0.080	0.077
0.099	0.179	0.067	0.071
0.091	0.076	0.072	0.097
0.084	0.164	0.072	0.079
0.059	0.103	0.090	0.071
0.084	0.102	0.067	0.069
0.099	0.175	0.090	0.078
0.095	0.121	0.134	0.083
Mean 0.089	**Mean 0.134**	**Mean 0.092**	**Mean 0.077**

Pigment results

$_{L}$-tyrosine dependent pigment production was progressively from evaluated, and the increase of pigment was dependent on the $_{L}$-tyrosine, where $_{L}$-tyrosine-supplemented strains produced pigment in 20th, 40th and 50th hours (Tables 3, 4 and 5). As a result, $_{L}$-tyrosine-supplement increased pigment production in *V. alginolyticus*. Pigment increase was measured by determining the absorbance at 405 nm wavelength using a spectrophotometer (Table 2). Positive control *C. violaceum* 12472 strainwas rejuvenated in special medium. Pigment was found in *V. alginolyticus* (Figure 4).

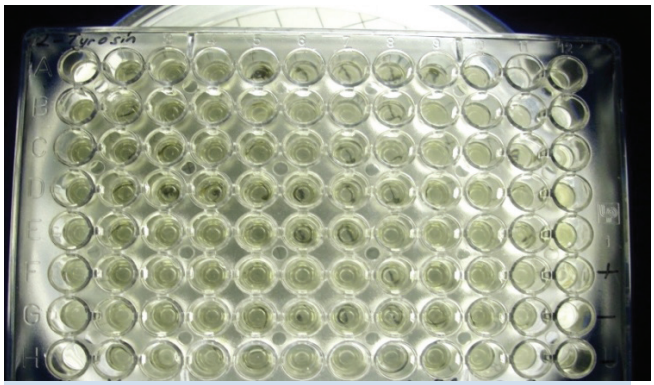

Figure 4. Pigment production in *V. alginolyticus* (20th hour) (yellow pigment)

Table 2. Optical density measurements of pigment in strain at a wavelength of 405 nm for *V. alginolyticus* (monitoring the pigment increase of strains)

Strain	Strain cod	20th hour	40th hour	50th hour
V. alginolyticus	ATCC	↑	↑	↑
V. alginolyticus	V5	↑	↑	↑
V. alginolyticus	V6	↑	↑	↑
C. violaceum	12472 (positive control)	↑	↑	↑

Table 3. Optical density measurements of pigment in strain at a wavelength of 405 nm for *V. alginolyticus* (20th hour)

Strain CFU/mL	20th hour *V. alginolyticus* ATCC	20th hour Added-$_{L}$-tyrosine *V. alginolyticus* ATCC	20th hour *V. alginolyticus* V5	20th hour Added-$_{L}$-tyrosine *V. alginolyticus* V5	20th hour *V. alginolyticus* V6	20th hour Added-$_{L}$-tyrosine *V. alginolyticus* V6	20th hour *C. violaceum* 12472	20th hour Added-$_{L}$-tyrosine *C. violaceum* 12472
10^7	0.221	0.245	0.211	0.235	0.210	0.241	0.310	0.333
10^7	0.218	0.249	0.205	0.248	0.212	0.237	0.311	0.328
10^7	0.209	0.248	0.205	0.251	0.205	0.241	0.259	0.319
10^7	0.207	0.251	0.195	0.252	0.200	0.249	0.308	0.331
10^7	0.211	0.252	0.201	0.257	0.203	0.240	0.320	0.329
10^7	0.222	0.249	0.215	0.246	0.219	0.244	0.302	0.332
10^7	0.221	0.243	0.212	0.253	0.218	0.239	0.300	0.337
10^7	0.219	0.257	0.213	0.250	0.220	0.251	0.301	0.338
	Mean 0.216	**Mean 0.249**	**Mean 0.207**	**Mean 0.249**	**Mean 0.210**	**Mean 0.242**	**Mean 0.301**	**Mean 0.330**

Table 4. Optical density measurements of pigment in strain at a wavelength of 405 nm for *V. alginolyticus* (40th hour)

Strain CFU/mL	40th hour *V. alginolyticus* ATCC	40th hour Added-$_{L}$-tyrosine *V. alginolyticus* ATCC	40th hour *V. alginolyticus* V5	40th hour Added-$_{L}$-tyrosine *V. alginolyticus* V5	40th hour *V. alginolyticus* V6	40th hour Added-$_{L}$-tyrosine *V. alginolyticus* V6	40th hour *C. violaceum* 12472	40th hour Added-$_{L}$-tyrosine *C. violaceum* 12472
10^7	0.209	0.259	0.205	0.261	0.199	0.243	0.299	0.354
10^7	0.223	0.250	0.219	0.249	0.220	0.248	0.310	0.360
10^7	0.229	0.248	0.212	0.268	0.224	0.253	0.305	0.362
10^7	0.220	0.261	0.199	0.256	0.203	0.260	0.318	0.363
10^7	0.221	0.254	0.191	0.259	0.211	0.241	0.329	0.359
10^7	0.202	0.258	0.203	0.261	0.213	0.234	0.310	0.369
10^7	0.216	0.260	0.189	0.251	0.216	0.247	0.301	0.357
10^7	0.231	0.255	0.198	0.246	0.222	0.242	0.305	0.369
	Mean 0.218	**Mean 0.255**	**Mean 0.202**	**Mean 0.256**	**Mean 0.213**	**Mean 0.246**	**Mean 0.309**	**Mean 0.361**

Table 5. Optical density measurements of pigment in strain at a wavelength of 405 nm for *V. alginolyticus* (50th hour)

Strain CFU/mL	50th hour *V. alginolyticus* ATCC	50th hour Added-L-tyrosine *V. alginolyticus* ATCC	50th hour *V. alginolyticus* V5	50th hour Added-L-tyrosine *V. alginolyticus* V5	50th hour *V. alginolyticus* V6	50th hour Added-L-tyrosine *V. alginolyticus* V6	50th hour *C. violaceum* 12472	50th hour Added-L-tyrosine *C. violaceum* 12472
10^7	0.201	0.263	0.233	0.261	0.229	0.252	0.300	0.341
10^7	0.213	0.259	0.241	0.243	0.245	0.260	0.311	0.360
10^7	0.209	0.265	0.235	0.249	0.237	0.254	0.291	0.343
10^7	0.212	0.261	0.241	0.244	0.239	0.271	0.301	0.339
10^7	0.206	0.270	0.240	0.251	0.254	0.261	0.299	0.342
10^7	0.203	0.265	0.238	0.258	0.237	0.270	0.302	0.333
10^7	0.208	0.264	0.234	0.252	0.240	0.250	0.298	0.330
10^7	0.209	0.260	0.230	0.257	0.249	0.258	0.301	0.334
	Mean 0.207	**Mean 0.263**	**Mean 0.236**	**Mean 0.251**	**Mean 0.241**	**Mean 0.259**	**Mean 0.300**	**Mean 0.340**

Table 6. Optical density measurements of elastase in strain at a wavelength of 630 nm for *V. alginolyticus*

Strain CFU/mL	*V. alginolyticus* ATCC	*V. alginolyticus* V5	*V. alginolyticus* V6	*P. aeruginosa* PAO1
10^7	0.411	0.391	0.433	0.285
10^7	0.395	0.405	0.437	0.291
10^7	0.419	0.409	0.429	0.289
10^7	0.399	0.399	0.449	0.301
10^7	0.415	0.419	0.447	0.271
10^7	0.417	0.404	0.431	0.305
10^7	0.408	0.397	0.432	0.307
	Mean 0.409	**Mean 0.403**	**Mean 0.436**	**Mean0.292**

Elastase results

V. alginolyticus was produce elastase. *V. alginolyticus* exhibited high levels of elastase. Elastase was measured by determining the absorbance at 630 nm wavelength using a spectrophotometer (Table 6). *V. alginolyticus* V6 strain was found the strongest elastase with an OD value of 0.436 and the elastase strength of this strain was stronger than *P. aeruginosa*.

Amylase results

Amylase which one of virulence under management of QS and which effective in disease was determined production at *V. alginolyticus*.

Rhamnolipid results

V. alginolyticus was not produce rhamnolipid.

Protease results

The results of the protease tests for bacteria, *V. alginolyticus* strains were negative and showed no protease.

Hemolysis results

Hemolysis was not detected in *V. alginolyticus*. Zone diameters of *V. alginolyticus* that was not cause hemolysis on blood agar after 24 hours of incubation was recorded as zero, “0 mm”.

Table 7. Summary of the QS.Overview table. AHL profiling of *V. alginolyticus* and detection of *V. alginolyticus* virulence factors

Strain	Strain cod	Strain property	AHL molecules	Biofilm	Pigment	Elastase	Amylase	Rhamnolipid	Protease	Haemolysis
V. alginolyticus	ATCC	Tested strain	OdDHL	+	+	+	+	-	-	-
V. alginolyticus	V5	Tested strain	OdDHL	+	+	+	+	-	-	-
V. alginolyticus	V6	Tested strain	OdDHL	+	+	+	+	-	-	-
*P. aeruginosa**	PAO1	Reference strain	BHL/ OdDHL	+	+	+	+	-	-	-
*C. violaceum***	CV026	Marker strains	BHL							
*A. tumefaciens***	NT1	Marker strains	OdDHL							
*C.violaceum****	12472	Reference strain			+					

* Strain used as positive control strain

** Strains used as marker strains for AHL molecules detection

*** Strains used as a positive control strain for pigment assay

Based on the findings presented in this study, it is clear that QS has a role in management the production of virulence factors in *V. alginolyticus*. AHLs are involved in many key regulations and play crucial roles in the progress of Vibrio diseases. The results obtained in this study revealed that cell to cell communication and QS managements the production of certain virulence factors (Table 7). It was determined that all *V. alginolyticus* strains study produced OdDHL signal molecule and produced virulence, such as biofilm, pigment, elastase, and amylase cause critical effects in diseases via this molecule.

DISCUSSION

Several virulences and cell density-dependent QS pathways were investigated in *V. alginolyticus*. Much evidence has shown that sRNAs have evolved bacteria fine-tune gene expression together with other transcriptional factors (Nitzan et al., 2017; Dutta and Srivastava, 2018). Liu et al., (2020b) first conducted research that attempted to identify a cell density-dependent sRNA, Qrr, in *V. alginolyticus*. *C. violaceum,* which was used widely in microbiology labs, involved QS research (Kothari et al., 2017). Nurcan in 2010, QS investigated in some gram-negative fish pathogenic bacteria and reported this bacteria communication. Cross-feeding results showed that no bacterial suspension induced visible violacein production in *C. violaceum* CV026, indicating a lack of short side-chain BHL production by the tested *V. alginolyticus* strains. With micro-biological monitoring systems, our research has also shown that *V.alginolyticus* did not produce BHL signaling molecule; however, produced OdDHL signaling molecule. With detection of OdDHL, presence of QS in *V. alginolyticus* was evidenced.

Ye et al., (2008)was identified *V. alginolyticus* luxS homologue. It was also shown that *V. alginolyticus* produced AI-2 which might be associated with potential luxS-dependent QS. By constructing the *LuxS* null mutants of *V. alginolyticus*, its *LuxS*/ AI-2-dependent QS was further examined. Data from biofilm and flagella strongly indicated that *V. alginolyticusLuxS* QS played essential role in pathogenicity (Ye et al., 2008). Indeed, previous studies have shown that AHLs increases the survival of marine Vibrio by regulating key processes of biofilm formation in many ways (McDougald et al., 2006). First, AHLs regulate the excretion of Extracellular Polymeric Substances (EPS) to constitute the cage construction of biofilm matrix. The matrix provides a suitable space for bacterial colonization and stable metabolism. In our study, *V. alginolyticus* proved interbacterial communication and biofilm.

Bruhn et al. 2005, 59 strains, representing nine pathogens, were tested against 2 AHL marker bacteria (*A. tumefaciens* NT1 and *C. violaceum* CV026) well diffusion assay and by thin-layer chromatography. Strainswere further characterized by high performance liquid chromatography-high resolution mass spectrometry. AHLs were produced by all strains of *Vibrio salmonicid* and *Vibrio vulnificus*. OHHL and HHL were detected in *V. salmonicida*. In conclusion, the production of AHL was common among the examined strains (Bruhn et al., 2005). Bacterial pathogens, QS has been shown to regulate the production of virulence determinants (Williams et al., 2000). Similarly, OdDHL signal molecule was detected in *V. alginolyticus* and production of virulence, such as biofilm, pigment, amylase, elastase via OdDHL under QS management was determined. AHL acts as a key regulator biofilm in *P. aeruginosa* and pigment in *C. violaceum* ATCC 12472 (Rashiya et al., 2021). In our study, *P. aeruginosa* and *C. violaceum* ATCC 12472 were used as a control strain. Biofilm and pigment were determined in *V. alginolyticus*. Pigment increased continuously at all measurement hours 20^{th}, 40^{th} and 50^{th}.

Motility and biofilm production have been managed by QS in *V. alginolyticus* (Gu et al., 2016). *V. anguillarum* produces biofilm-associated slime (Filik, 2019; Filik and Kubilay, 2019). Similar to results of these researchers our findings showed that V. alginolyticus produced biofilm, pigment, elastase and amylase under the management QS. QS induced gene expression of *Asp*, protease from *V. alginolyticus* (Chang and Lee, 2020)**.**Unlike the findings in study of Chang and Lee 2020, it was determined that **V. alginolyticus** no produce protease in our study.

Oligopeptide permease (Opp) was proven to play a variety of important roles in virulence in bacteria. In their previous research, Liu et al.,(2017) revealed that the *opp* gene cluster was identified in *V. alginolyticus* with transcriptome sequence, indicating that Opp might play roles, such as adhesion. Haemolysin is an agent important in pathogenesis (Syed et al., 2009). Wu et al., (2007) found *oppA* haemolysis, while function *oppBCDF* haemolysis regulation was not verified. Liu et al., (2017) reported that *oppABCDF* was stable silenced in *V. alginolyticus* and haemolysis was

detected.Our research showed that *V. alginolyticus* did not produce haemolysis. However, in addition, Liu et al., (2017) indicated that *oppABCDF* contributed to multistep of *V. alginolyticus* pathogenesis, biofilm and haemolysis. Study of Liu et al., (2017) comparison with our research findings, *V. alginolyticus* produced biofilm, however was not found hemolysis.

Dermatopathogenicity of *V. alginolyticus* was analyzed for the presence of virulence represented by three enzymes collagenolytic: elastases, collagenases and chondroitinases. Moreover, it was observed that the production of three enzymes from *V. alginolyticus* wasconsidered the main virulence of bacteria, especially in cases of dermatological infection (Lafisca et al., 2008). *V. alginolyticus* also shows pathogenic effects in humans at the same time. There are many case reports regarding this. (Baran et al., 2015). Elastases are related with human pathogenicity. In our research, *V. alginolyticus* was found to produce elastase. Surprisingly, the detection of elastase strain-specific to human pathogen *P. aeruginosa* in *V. alginolyticus* was considered a significant finding.

Liu et al., 2016, the discovery of novel multifunctional enzyme Amy63 produced by *V. alginolyticus* 63 was reported and remarkably; Amy63 possessed amylase, agarase and carrageenase. Amy63 substrated promiscuous α-amylase, with the substrate priority order of starch, carrageenan and agar.Likewise, *V. alginolyticus* produced amylase.

Rhamnolipids were formed *via* QS in *P. aeruginosa* (Nickzad et al., 2015)and *A. hydrophila* (Filik, 2020; Filik and Kubilay, 2020). However, rhamnolipid was not found in *V. alginolyticus*.

Hemolysin is one of the major pathogenic factors in *V. alginolyticus*, which shows hemolytic effect. It is associated with different *V. alginolyticus* that manifest either wound or intestinal infection as their clinical symptom (Mizuno et al., 2019). In contrast, hemolysis was not detected in study.

The fact that virulence has not produced at all in this study strains suggests that strains may have mutations in different parts of system genes. Mutations explain defects virulence in strains. 827 strains were screened as quorum sensing inhibitor (QSI) compounds by Reina et al., 2019. Selected *V. alginolyticus* as QSI. QSI of these molecules was found to significantly inhibit *C. violaceum* 12472 virulence, *P. aeruginosa* swarming and twitching (Reina et al., 2019). In this study, it was not QSI role of *V. alginolyticus*,but QS in *V. alginolyticus* and virulence managed by QS were screened. It was found to produce biofilm, pigment, amylase, elastase, but not rhamnolipid, hemolysis protease. These research findings will guide future *V. alginolyticus*' QS identification and investigation of its QSI potential.

V. alginolyticus apart from a fish and and aquatic invertebrate pathogen, is also a food born pathogen occasionally affecting humans by causing food-poisoning (Fu et al., 2016). *V. alginolyticus* also causes infections in humans (Schmidt et al., 1979). Since *V. alginolyticus* is both a major disease agent and a foodborne pathogen in humans, it occasionally appears as a cause of food poisoning. Therefore, it is a pathogen with a very high risk of contamination and capacity. The fact that the contamination network covers such a large area is due to the strong bacterial communication system (QS).

V. alginolyticus represents a severe pathogen that raises public concerns and causes huge economic loss in aquaculture (Gong et al., 2020). Spirulina platensis has been tried to reduce the pathogenicity of *V. alginolyticus* in aquaculture (Tayag et al., 2018), and other studies are still underway to reduce its virulence potency. Membrane-disruptive engineered peptide amphiphiles restrain the proliferation of penicillins and cephalosporins resistant *V. alginolyticus* and *Vibrio parahaemolyticus* in instant jellyfish (Zeng et al., 2022). We opinion that our research is an important source of literature among these studies conducted to stop *V. alginolyticus* infection, which causes great economic losses in aquaculture.

The communication system coordinates bacterial behavior and expression of genes in response to population density (Rémy et al., 2018). As the list of bacteria that employ *QS* continues to grow, so does the number of possibilities for exploiting these regulatory mechanisms. Because many important pathogens use *QS* to regulate virulence, strategies designed to interfere with signaling systems will likely have broad applicability for the disease control. In the future, it will be intriguing to investigate whether additional pathogens utilize *QS* as a part of their pathogenic lifestyle and, if so, whether the production of AHL can be exploited to control infections (Deep et al., 2011).

It can be envisaged that virulence is important for the infection and transmission cycle of pathogenic bacteria and new non-antibiotic based treatments,

which directly inhibit virulence rather than the bacteria growth. An advantage of such an approach would be that bacteria do not develop resistance since they are neither killed, nor their growth is inhibited. In this context, an understanding of virulence is absolutely central (Bruhn et al., 2005).

Molecular studies showed that *V. alginolyticus* could acquire virulence genes by transduction between other bacterias. Then, *V. alginolyticus* can provide reservoir for virulence genes that are present in the environment. Despite these premises, the studies conducted about QS and virulence are inadequate, which has remained under-researched. It was reported that researchers' increased knowledge of this bacteria of QS system and under the management virulence, specificity of symptoms was correlated to it and its susceptibility to many antibiotics surely participated scarcity of vibriosis cases. The presence of virulence genes should be a perspective in future studies in order to evaluate virulence of *V. alginolyticus*. On the condition that AHL molecules regulate the expression of virulence in bacteria, as shown to occur in some bacterial pathogens, novel disease control measures may be developed by blocking AHL-mediated communication and suppressing virulence.

V. alginolyticus has been shown to contain multiple quorum-sensing circuits. When all these situations are evaluated, it is will effective to stop QS while bacteria do not communicate until there is no time for disease. Detection and destruction of signal molecules during communication of *V. alginolyticus* will result in more successful profile than existing methods for in early diagnosis of diseases. In conclusion, it has been observed that disrupting the QS of the pathogen can result in a significant decrease in virulence. Understanding how bacteria communicate with each other has a number of important implications for the control of pathogens, and for the screening and exploitation of bacteria that produce antibiotics. Since many important fish pathogens use quorum sensing to regulate virulence, strategies intended to interfere with their signalling systems will likely have many potential applications. The disruption of signalling systems offers an opportunity to prevent the bacteria from responding to the signal and thereby prevent the expression of virulence factors. Biotechnological research is now focused on the development of AHL antagonists. Disruption of signalling systems (Quorum Quenching, QQ) offers an opportunity to prevent bacteria from responding to signal and thereby prevent the expression of virulence. QQ refers to the mechanism by which bacterial communication can be interrupted. By using QQ, these systems can be blocked, thereby reducing the pathogenicity of *V. alginolyticus*. It would be beneficial to study the possibility of using quorum quenchers to reduce the side effects that result from overusing antibiotics by interrupting the communication between *V. alginolyticus*. QQ can be achieved by inhibiting the production of auto-inducers, their detection by receptors, or their degradation. Certainly, improved understanding and potential applications of AHL-based QS and QQ will help to fish diseases treatment systems successfully. This situation will also mark an era the treatment.

CONFLICT OF INTEREST

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

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