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Molecular Characteristics Demonstrate the Occurrence of Phylogenetic Similar Isolates of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in Aquatic Environments

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Abstract

Vibrio alginolyticus is an opportunistic fish pathogen with a potential to cause septicemia and often associated with other *Vibrio* infections, particularly *Vibrio parahaemolyticus*, and can pose a significant threat to aquatic health. This study investigated the biochemical and phylogenetic characteristics of *V. alginolyticus* isolated from various aquaculture farms, focusing on its prevalence, hemolysin genes, genetic relatedness, and antibiotic susceptibility. Out of 92 brackish water aquaculture farm samples, 16 isolates were biochemically confirmed as *V. alginolyticus*, with 12 subsequently confirmed by polymerase chain reaction targeting collagenase gene. Molecular analysis of the thermolabile hemolysin gene (*tlh*) via specific PCR amplified a 450 bp fragment in 8 isolates, confirming the presence of the *tlh* gene. Pulsed Field Gel Electrophoresis (PFGE) typing differentiated *tlh*-positive and *tlh*-negative *V. alginolyticus* isolates with 92% genetic similarity. The isolates exhibited proteolytic, lipolytic, and lecithinase activities. Notably, the isolates showed intermediate resistance to most of the tested antibiotics, indicating exposure to antimicrobial agents. This study provides evidence of the presence of similar phylogenetical isolates of *V. alginolyticus* and *V. parahaemolyticus* in aquatic environments. Furthermore, this study emphasizes the importance of expanding surveillance programs by incorporating strain-specific characterization to better understand and control vibriosis in aquaculture.

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Introduction

The members of the genus *Vibrio* are the most important food-borne and aquatic pathogens which are responsible for illness in humans and cause large scale mortalities in the aquaculture sector (Feldhusen, 2000). Next to *V. parahaemolyticus, V. alginolyticus* has the potential to cause disease in marine aquaculture industry, especially in shellfish and crustaceans (Liu et al., 2016). *V. alginolyticus* is a Gram-negative marine bacterium having zoonotic potential for animals and humans. Previously it was assembled and termed as *V. parahaemolyticus* biotype 2 due to their common morphological and phenotypical reactions (Deng et al., 2016). The ability to ferment sucrose by *V. alginolyticus* is the major difference in the biochemical reaction between *V. parahaemolyticus* and *V. alginolyticus*. Therefore, the application of PCR is necessary for the confirmation and identification of the species *V. alginolyticus*, as there is a chance of cross-reaction with *V. parahaemolyticus* while screening for genes encoding the hemolysin (Di Pinto, Ciccarese, Tantillo, Catalano, & Forte, 2005). Even though this organism stands next to *V. parahaemolyticus* in terms of intensity of virulence, their occurrence may cause considerable economic loss in aquaculture (Takemura, Chein, & Polz, 2014).

In the international trade of marine fishes, screening of *Vibrio* species has become a criterion of microbiological testing. Even though *Vibrio* species are a common inhabitant of aquatic environment, some species are emerging as pathogens which can cause up to more than 50% mortality of all clinical cases

(Bisharat et al., 1999; Daniels & Shafaie, 2000). Several reports are available which shows *V. alginolyticus* associated with infections in humans. (Levine, Griffin, & Gulf Coast Vibrio Working Group, 1993; Gomez, Fajardo, Patiño, & Arias, 2003; Aguirre Guzmán, Ruíz, & Ascencio, 2004). The virulent strains of *V. alginolyticus* are considered as an emerging strain that carry the *trh* gene that is homologous to that in *V. parahaemolyticus*. However, many strains of *Vibrio alginolyticus* are avirulent and can be used as probiotic strains (Marhual et al., 2012). There exists wide variation among strains that differs between harmful and beneficial strains which paves way to control infection. The *tlh* gene is considered a species-specific marker in *V. parahaemolyticus* and the pathogenic strains possess either or both *trh* and *tdh* genes (Bej et al., 1999). Yáñez et al. (2015) reported *tlh* amplicon in *V. aliginolyticus* isolated from shellfish samples from Antofagasta, which showed 81% similarity with *tlh* gene of *V. parahaemolyticus*.

In view of the above facts the present study aimed to isolate *V. alginolyticus* from different aquaculture farms to study the biochemical characteristics, thereby to determine their prevalence in fish and aquaculture farm environment. Further, the study aimed to understand the phylogenetic similarities between *V. parahaemolyticus* and *V. alginolyticus* by investigating the presence and variation of species and pathogenic specific gene fragments among them. The antibiotic susceptibility and genetic relatedness of the species were also taken under study.

Materials and Methods

A total of 92 samples (fish -42, shrimp -30, water -12 and farm sediment -8) were collected from brackish water aquaculture ponds, and hatcheries in and around Cochin, Kerala, India. The isolation and biochemical of *Vibrio* sp. were carried out as per the BAM protocol for Vibrios (FDA, 2004) with slight modification. Briefly, all the presumptive isolates from Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar plates were streaked onto Chromogenic Vibrio (CV) agar (Himedia, India) and cream white coloured colonies were selected for further biochemical identification. Gram negative isolates showing positive reaction to oxidase, catalase, lysine decarboxylation, ornithine decarboxylation, D mannose fermentation and mannitol fermentation, voges proskauer test and salt tolerance upto 10% NaCl, negative reaction to arginine decarboxylation, lactose, arabinose and cellobiose fermentation, with no growth at 0% salt were selected for further characterization.

Molecular level confirmation was carried out by PCR using species- specific genes with primers targeting the collagenase gene (Di Pinto et al., 2005). DNA lysate from *V. alginolyticus* ATCC 17749 was used as the positive control.

Amplification of hemolysin genes such as *tlh, trh,* and *tdh* genes in *V. alginolyticus* isolates was screened by employing specific primers as described by Bej et al. (1999). *V. parahaemolyticus* ATCC17802 was used as a reference strain for the amplification of *tlh* and *trh* gene and *V. parahaemolyticus* O3:K6 strain (NICED, Kolkata) was used as positive control for *tdh* gene.

Molecularly confirmed isolates were checked for the secretion of extracellular products such as protease, lipase, hemolysin, lecithinase, (Nakazawa, Yamada, & Ishibashi,1987; Esselmann & Liu, 1961) activities on trypticase soya agar (TSA) media supplemented with 1% NaCl containing, 1% (w/v) skim milk, 1% (v/v) Tween 80, 5% Sheep red blood cells and 1% (v/v) egg yolk emulsion, respectively. Agar plates were then observed for zone formation by incubating at 35 + 2°C for 2-3 days.

PFGE was carried out as per the pulse net procedure for performing PFGE of *V. cholerae* and *V. parahaemolyticus* (Parsons et al., 2007). The identified *V. alginolyticus* isolates were inoculated into Luria Bertani broth supplemented with 2% NaCl and kept overnight at 35 + 2ºC. The freshly grown culture was centrifuged at 10,000 rpm for 3 min and the pellet was dissolved in 1X phosphate buffered saline (PBS) and the optical density (OD_{610}) was adjusted to 0.8 to 1.0. The agarose plugs were prepared in disposable plug molds (Amersham Biosciences, USA) by mixing and transferring 1% agarose with an equal volume of bacterial cell suspension containing 20 µl proteinase K (20 mg ml^{-1}) to disposable plug molds. The plugs were allowed to set for 20-30 min. After the set time, plugs were carefully removed using spatula and transferred to the cell lysis buffer. Then, the plugs were washed initially with preheated double distilled water and then with TE buffer for 4-5 times each for 10-15 minutes interval in a shaking water bath of temperature 54-55°C. The washed plugs were cut into small slices of size 2 - 2.5 mm. The restriction

digestion was done using 10 U of *NotI* restriction enzyme for 4 h at 37 °C. Then the plugs were removed from the digestion solution and inserted into 1% agarose gel carefully without formation of any air bubbles and sealed with melted agarose. The gel was taken to electrophoresis system (CHEFF MAPPER, Bio-rad Laboratories, USA) and run for 18 h. Gel images were captured in a Gel documentation system (Bio-Rad, USA).

The PFGE gel photographs were analysed using Bionumerics 7.6 (Applied Maths, New York) software to determine the level of similarities between the PFGE patterns. The dendrogram was constructed using UPGMA (unweighted pair group mean analysis) based on Sorensen-Dice similarity coefficient with 1% tolerance level.

Antibiotic susceptibility of *V. alginolyticus* was determined by disc diffusion method on Mueller Hinton agar (MHA), supplemented with 1% NaCl by following the guidelines of Clinical and Laboratory Standards Institute (Wayne, 2017). Briefly, colonies of pure culture from nutrient agar were selected and swabbed using a sterile cotton swab. The colonies were then transferred to sterile 0.85% PBS, and the turbidity was adjusted to the 0.5 McFarland standards. The PBS suspension was then spread plated on MH agar. The inoculated plates are allowed to dry for 2 to 3 min at room temperature. The antibiotic discs (Himedia, India) used were tetracycline (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), aztreonam (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ampicillin (10 μg), cefotaxime (30 μg), cefpodoxime (10 μg) and polymyxin (300 units). The discs were dispensed onto well-labeled inoculated (MHA) plates using disc dispenser. Then the plates were incubated at 35 + 2°C for 24 h for measuring the zone of inhibition. The test was carried out in duplicate.

Results and Discussion

A total of 92 samples were screened, and sucrose positive colonies were detected from 86 samples. The large yellow mucoid colonies (n=178) from TCBS plates were selected as presumptive isolates of *V. alginolyticus.* All the fish and shell fish samples were positive for presumptive identification. Further streaking to CV agar plates reduced the presumptive isolates to 68, out of which only 16 isolates representing 16 samples were confirmed biochemically as *V. alginolyticus.* All the confirmed isolates formed colourless colonies on CV agar plates. A total of 62.5% of the isolates showed salt tolerance up to 10 % level of NaCl and other isolates have shown salt tolerance up to 8% NaCl level. Further all the confirmed isolates yielded characteristic biochemical reactions.

The main difficulty faced by using conventional medium (TCBS) for the isolation of pathogenic *Vibrio* species is the low discrimination ability for the differentiation of *Vibrio* species; as recommended by standard NF ISO 8914:1991 and World Health Organization. False negative and positive results hampered the clear identification of *Vibrio* species. Fabbro, Cataletto, and Negro, (2010) have stated the accurate biochemical identification of environmental strains is often difficult compared to clinical strains due to the ability of growth of other marine microorganisms (*Aeromonas* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Pasteurella* sp. and *Agrobacterium* sp.) on TCBS agar. Sucrose fermenting bacteria such as *V. cholerae, V. furnissii* and *V. fluvialis* also produce yellow-colored colonies which lead to difficulty in differentiating from *V. alginolyticus* colonies on TCBS agar. CHROM agar *Vibrio* medium has been used for the isolation of certain strains of *Vibrios* especially *V. parahaemolyticus*, *V. cholerae* and *V. alginolyticus* based on the β-glucosidase and β-galactosidase activity (Yeung & Thorsen, 2016).

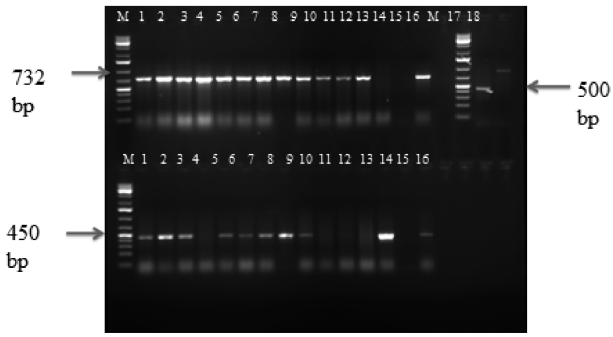

Fig. 1. Detection of *collagenase* gene (732 bp), amplification of *trh* gene (500bp), *tlh* gene (450bp) in *V. alginolyticus* isolates.

Top: Lane M: DNA ladder (100 bp plus), Lane1: *V. alginolyticus* ATCC 17749; Lane 2-15: *V. alginolyticus* isolates from different samples. Lane 16: *V. parahaemolyticus* ATCC 17802; Lane 17: *V. alginolyticus* ATCC 17749; Lane 18: *V. alginolyticus* isolate

Bottom: Lane M: DNA ladder (100 bp plus), Lane1: *V. parahaemolyticus* ATCC 17802; Lane 2-15: *V. alginolyticus* isolates; Lane 16: *V. alginolyticus* ATCC 17749

Several similarities in biochemical reaction exists in both *V. alginolyticus* and *V. parahaemolyticus*. Studies have shown that several phenotypical and molecular characteristics of *V. parahaemolyticus* and *V. alginolyticus* were similar with 99.8% identical which make difficulty in differentiation of the isolates (Osorio & Klose, 2000; Croci et al., 2007). However, in this study, the biochemical characteristics of *V. alginolyticus* isolates showed similar results prescribed by Bacteriological Analytical Manual for the isolation and identification of *Vibrio*s (FDA, 2004) except in the salt tolerance test. In contrast to these results, Montieri, Suffredini, Ciccozzi, and Croci (2010) reported statistically no significant difference in the salt tolerance test among *V. alginolyticus* isolates.

PCR assay was conducted to determine the species-specific collagenase gene in biochemically confirmed *V. alginolyticus* (n=16) resulting in the amplification of the expected amplicon size of 737 bp in 12 isolates (Fig. 1). The variation in collagenase sequences of different *Vibrio* species such as *V. parahaemolyticus, V. alginolyticus* and *V. cholerae* were utilized for developing genetic markers for species discrimination (Di Pinto et al., 2005). The collagenase activity was found in *V. alginolyticus* and has very low neurotoxic activity compared to other bacterial enzymes. It appears to play a vital role in tenderization of meat as well as wound healing (Takeuchi et al., 1992).

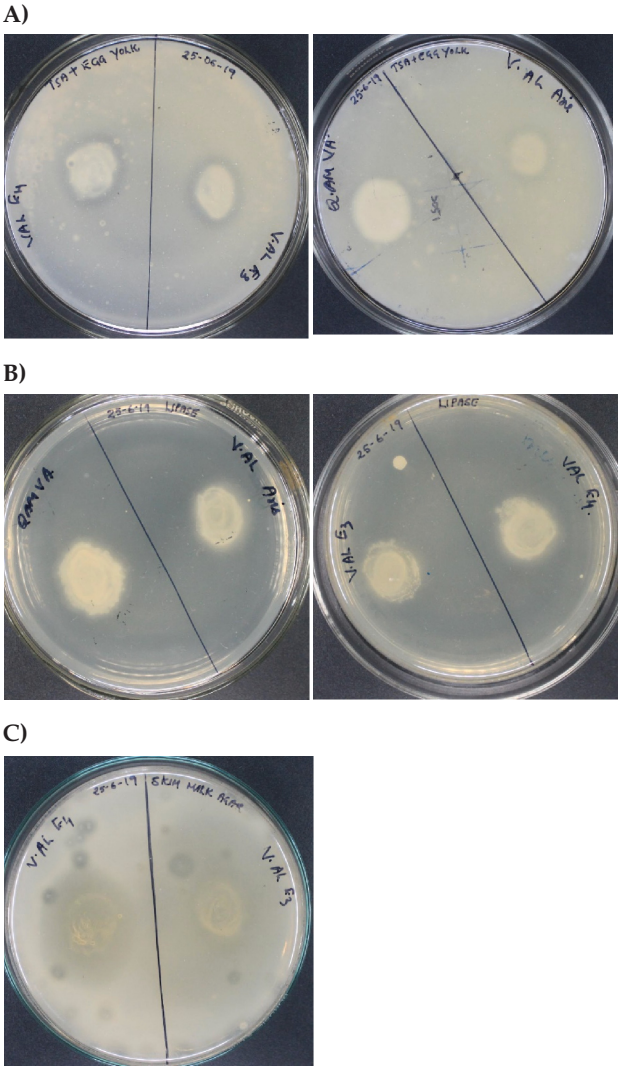

Fig. 2. *V. alginolyticus* isolates showing different activities on various media A) Lecithinase activity on Trypticase soya agar with 1% (v/v) egg yolk; B) Lipase activity on Trypticase soya agar with 1% (v/v) Tween 80 C). Protease activity on Trypticase soya agar with 1% (v/v) skimmed milk

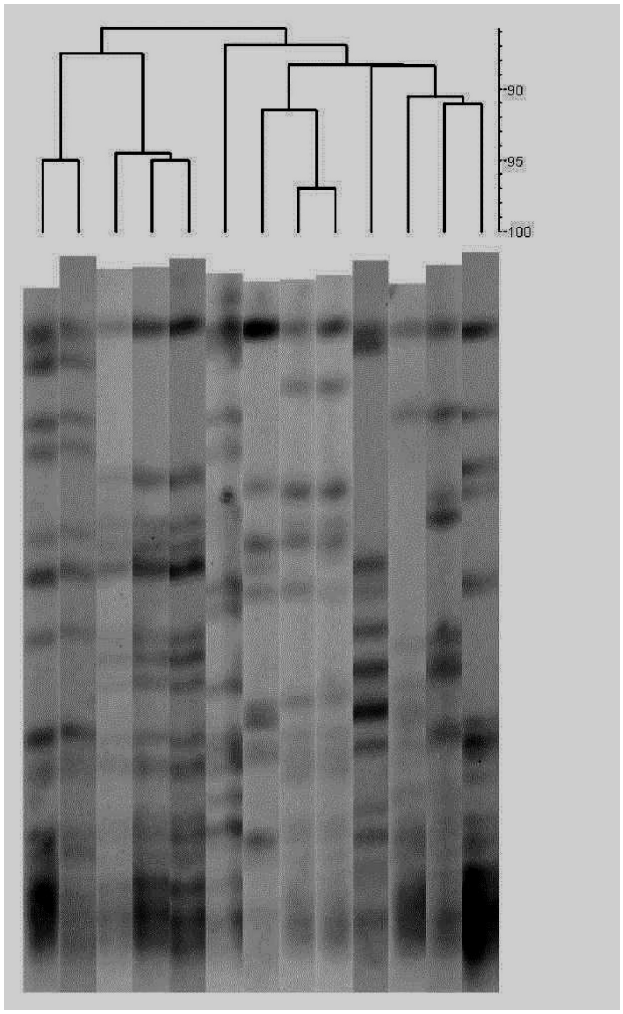

Fig. 3. Dendrogram and PFGE banding pattern of *Not1*-digested chromosomal DNA of *V. alginolyticus* isolates

Hemolysin genes such as *tlh, trh* and *tdh* genes are known for *V. parahaemolyticus* as specific and pathogenic specific markers and are widely used for the surveillance of these bacteria from both environmental and clinical sources (Bej et al., 1999). The presence of *tlh* gene in *V. alginolyticus* can be expected due to the several phenotypical similarities of both *V. parahaemolyticus* and *V. alginolyticus* strains. Reports have been shown that *tlh* gene is widespread among *Vibrios* such as *V. alginolyticus, V. harveyi, V. fischeri, V. proteolyticus* etc (Wang, Liu, Ma, Rui, & Zhang, 2007).

A total of 12 *V. alginolyticus* isolates were screened for *tlh* gene and the gene was detected in 8 isolates (Fig. 1). Among them, one isolate was positive for *trh* gene in which amplicon size is above than the expected size of 500 bp. The amplification of *tlh* gene in *Vibrio* species may lead the inadequacy of the species-specific marker of *V. parahaemolyticus* in the event of surveillance of epidemiological investigations (Wang et al., 2007). In addition to this, several virulence related genes of *V. cholerae* and *V. parahaemolyticus* were reported in other *Vibrionaceae* family especially in *V. alginolyticus*, *V. mimicus*, *V. diabolicus* etc., (Klein, Pipes, & Lovell, 2018). Wang et al. (2007) reported that the *tlh* probe for *V. parahaemolyticus* lacks specificity due to its highly homologous nature to the hemolysin genes of *V. vulnificus* (*vvh).* This poses a significant concern when the sample becomes contaminated with other *Vibrio* species or when enrichment PCR is performed without selective enrichment and identification of the specific *Vibrio* species. This could lead to inaccurate conclusions and highlights the importance of proper sampling, selective enrichment, and identification techniques to ensure accurate results.

Exoenzyme activity on *V. alginolyticus* isolates showed clear zone formation in all the isolates in lecithinase, lipase and caseinase specific media while blood agar plates did not exhibit any zone formation in all tested isolates (Fig. 2). These exoenzymes are factors that possess pathogenicity to species.

In the present study, exoenzyme activity of *V. alginolyticus* was observed for the lecithinase, lipase and caseinase production. The result of this study suggests that the *V. alginolyticus* showed the production of lecithinase on egg yolk agar in 2-3 days. The zones around the organism were preceded by a zone of clearing of media which might have been due to its proteolytic activity. Lipase media and skim milk agar media also showed a zone of clearance in their respective media due to the production of lipase and caseinase by *V. alginolyticus*. The clearance zone around the colonies indicated that the isolates were slow producers of lecithinase (2 mm), lipase (below 2 mm) and protease (below 3 mm) production. Bunpa, Sermwittayawong, and Vuddhakul, (2016) studied 17 *V. alginolyticus* isolates obtained from environments, diseased fish and shrimp for the production of exoenzymes and reported that all of the isolates showed gelatinase, lecithinase and caseinase activities, while 75% of them possessed amylase and lipase activities. Interestingly, hemolytic activity was not shown by any of the isolates. It might be due to the absence of *tdh* activity in the isolates as several studies have shown that *tdh* controls various biological activities such as cytotoxic and hemolytic activity in *Vibrio* species (Wang, Hu, Deng, & Gooneratne, 2021). These extracellular enzymes were proposed to act as virulence factors for fish and other marine organisms by allowing the *Vibrio* species to adhere to the epithelial cells of the host, to evade the first barrier of natural defense and to colonise all internal organs.

All 12 isolates of *V. alginolyticus* were typed with *NotI* restriction enzyme with a high degree of genetic similarity (92%) and were divided into two pulse types A and B (Figure 3). Further, the pulse type A is subdivided into 5 subtypes A1, A2, A3, A4 and A5. The pulse type B is subdivided into 8 subtypes (B1, B2, B3, B4, B5, B6, B7, and B8). The isolates obtained from the same sampling location subtype B3 and B4 showed a unique pattern of 98% similarity in PFGE typing. All *tlh* positive isolates (n=8) formed unique pulse type B revealing the definite relationship between pulse types and the hemolysin gene of *V. alginolyticus.* All *V. alginolyticus* isolates lacking *tlh* gene forms pulse type A (A1, A2, A3 to A5). The standard culture of *V. alginolyticus* ATCC 17749 forms pulse subtype A4. However, the PFGE types and antibiotic resistance pattern didn't show any definite relationship.

PFGE is recognized as a gold standard of subtyping of food borne pathogens due to its sensitivity, specificity and reproducibility (de Boer, & Beumer, 1999). In this study, PFGE results showed a unique fingerprinting pattern for differentiating isolates of *V. alginolyticus* in terms of the hemolysin gene *(tlh* gene). The high level of genetic similarity in this

study may be due to the same geographic location in which the samples are drawn, implying closeness of the sampling locations (Mironova, Afanasev, Basov, Urbanovich, & Balakhonov, 2014). The identical banding pattern noted among the isolates such as A1 and A2, A4 and A5, B3 and B4, B6, B7, and B8 indicates that these isolates were closely related and may have originated from a single clone. Ren et al. (2013) examined the distribution and virulence gene detection in *V. alginolyticus* from different sources of mariculture systems and showed extensive genomic diversity and identical PFGE typing patterns in pathogenic isolates. However, a good correlation between PFGE clones and antimicrobial susceptibilities in different enteric pathogenic bacteria was reported previously by many workers (Liu et al., 2009; Du et al., 2018; Yang et al., 2019). Hence, it can be concluded that PFGE typing successfully differentiated *tlh* positive *V. alginolyticus* from *tlh* negative *V. alginolyticus* isolates from seafood.

The results of antibiotic resistance pattern showed that all isolates were sensitive to tetracycline and chloramphenicol (Table 1). Intermediate resistance was observed against ciprofloxacin (83.3%), aztreonam (8.3%), polymyxin (16.6%), ceftazidime (33%), cefpodoxime (16.6%), and cefotaxime (8.3%). Resistance to co-trimoxazole was shown by 16.6% of isolates. Resistance to ampicillin was found to be 100%. The gentamicin was associated with 50% of intermediate resistance, while the rest of the isolates were completely susceptible.

In this study, most isolates showed intermediate resistance that explains the isolates were exposed to antimicrobial agents. All the isolates were resistant to ampicillin and the intermediate resistance to most of the antibiotics tested showed the overuse of antibiotics in aquaculture operations for the treatment of diseases as well as for the growth promotion of fishes (Kang et al., 2016). Antibiotics resistant microorganisms are ecologically very important and this character is acquired either through the mutation or through the horizontal gene transfer mechanism. From the earlier studies, it was found that R-plasmid is responsible for antibiotics resistance character. Lee, Ab Mutalib, Law, Wong, and Letchumanan, (2018) reported that R-plasmids are widely distributed in *Vibrios* including *V. alginolyticus* and are responsible for the carrying of transferable drug resistance in these bacteria. They suggested that antibiotic resistant *V. alginolyticus* may survive better than sensitive organisms in surface water. The resistance of bacteria to antibiotics is attributed to the enzymatic destruction of antibiotic and the impermeability of the cell wall. Antibiotic susceptibility profiling helps to evaluate the efficiency of drugs used against various bacterial species that helps to correct strategic and tactic use of chemotherapy against bacterial infections (Larsen & Faird, 1980).

Conclusion

The present study concluded the prevalence of *tlh* positive *V. alginolyticus* was high (66%) compared to total *V. alginolyticus* (13.4%) from different brackish water aquaculture fish and farm samples PFGE typing showed a clear discrimination among *tlh* positive and *tlh* negative isolates. This indicates the inaccuracy of the epidemiological surveillance program of qualitative identification of *V. parahaemolyticus* using *tlh* gene. Hence, it will be considered as a serious concern especially in case of rapid detection by using direct enrichment PCR assay by targeting *tlh* gene.

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References

Aguirre Guzmán, G., Ruíz, H. M., & Ascencio, F. (2004). A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. *Aquaculture Research, 35*(15), 1395-1404. https://doi.org/10.1111/j.1365-2109.2004.01165.x.

Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C. L., Jones, D. D., & Kaysner, C. A. (1999). Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh, tdh* and *trh*. *Journal of Microbiological Methods, 36*(3), 215-225. https://doi.org/10.1016/S0167-7012(99)00037-8.

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodner, R., Cameron, D. N., Wykstra, D. L., Swerdlow, D. L., & Farmer, J. J. (1999). Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. *The Lancet, 354*(9188), 1421-1424. https://doi.org/10.1016/S0140-6736(99)02471-X.

Bunpa, S., Sermwittayawong, N., & Vuddhakul, V. (2016). Extracellular enzymes produced by *Vibrio alginolyticus* isolated from environments and diseased aquatic

animals. *Procedia Chemistry*, *18*, 12-17. https://doi.org/10.1016/j.proche.2016.01.002.

Croci, L., Suffredini, E., Cozzi, L., Toti, L., Ottaviani, D., Pruzzo, C., Serratore, P., Fischetti, R., Goffredo, E., Loffredo, G., Mioni, R., & *Vibrio parahaemolyticus* Working Group. (2007). Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology, 102*(1), 229-237. https://doi.org/10.1111/j.1365-2672.2006.03046.x.

Daniels, N. A., & Shafaie, A. (2000). A review of pathogenic *Vibrio* infections for clinicians. *Infections in Medicine*, *17*(10), 665-685.

de Boer, E., & Beumer, R. R. (1999). Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology*, *50*(1-2), 119-130. https://doi.org/10.1016/S0168-1605(99)00081-1.

Deng, Y., Chen, C., Zhao, Z., Zhao, J., Jacq, A., Huang, X., & Yang, Y. (2016). The RNA chaperone Hfq is involved in colony morphology, nutrient utilization and oxidative and envelope stress response in *Vibrio alginolyticus*. *PLoS One*, *11*(9), e0163689. https://doi.org/10.1371/journal.pone.0163689.

Di Pinto, A., Ciccarese, G., Tantillo, G., Catalano, D., & Forte, V. T. (2005). A collagenase-targeted multiplex PCR assay for identification of *Vibrio alginolyticus*, *Vibrio cholerae,* and *Vibrio parahaemolyticus*. *Journal of food Protection*, *68*(1), 150-153. https://doi.org/10.4315/0362-028X-68.1.150.

Du, Y., Wang, C., Ye, Y., Liu, Y., Wang, A., Li, Y., Zhou, X., Pan, H., Zhang, J., & Xu, X. (2018). Molecular identification of multidrug-resistant Campylobacter species from diarrheal patients and poultry meat in Shanghai, China. *Frontiers in Microbiology*, *9*, 1642. https://doi.org/10.3389/fmicb.2018.01642

Esselmann, M. T., & Liu, P. V. (1961). Lecithinase production by gram-negative bacteria. *Journal of Bacteriology*, *81*(6), 939-945. https://doi.org/10.1128%2Fjb.81.6.939-945.1961.

Fabbro, C., Cataletto, B., & Negro, P. D. (2010). Detection of pathogenic *Vibrio parahaemolyticus* through biochemical and molecular-based methodologies in coastal waters of the Gulf of Trieste (North Adriatic Sea). *FEMS Microbiology Letters*, *307*(2), 158-164. https://doi.org/10.1111/j.1574-6968.2010.01969.x.

Feldhusen, F. (2000). The role of seafood in bacterial foodborne diseases. *Microbes and Infection, 2*(13), 1651-1660. https://doi.org/10.1016/S1286-4579(00)01321-6.

Gomez, J. M., Fajardo, R., Patino, J. F., & Arias, C. A. (2003). Necrotizing fasciitis due to *Vibrio alginolyticus* in an immunocompetent patient. *Journal of Clinical Microbiology*, *41*(7), 3427-3429. https://doi.org/10.1128/jcm.41.7.3427-3429.2003.

Klein, S., Pipes, S., & Lovell, C. R. (2018). Occurrence and significance of pathogenicity and fitness islands in environmental *Vibrio*s. *AMB Express*, *8*. https://doi.org/10.1186/s13568-018-0704-2.

Lee, L. H., Ab Mutalib, N. S., Law, J. W. F., Wong, S. H., & Letchumanan, V. (2018). Discovery on antibiotic resistance patterns of *Vibrio parahaemolyticus* in Selangor reveals carbapenemase producing *Vibrio parahaemolyticus* in marine and freshwater fish. *Frontiers in Microbiology*, *9*, 2513. https://doi.org/10.3389/fmicb.2018.02513.

Levine, W. C., Griffin, P. M., & Gulf Coast Vibrio Working Group. (1993). *Vibrio* infections on the Gulf Coast: results of first year of regional surveillance. *The Journal of Infectious Diseases, 167*(2), 479-483. https://doi.org/10.1093/infdis/167.2.479.

Liu, F., Guan, W., Alam, M. J., Shen, Z., Zhang, S., Li, L., Shinoda, S., & Shi, L. (2009). Pulsed-field gel electrophoresis typing of multidrug-resistant *Vibrio parahaemolyticus* isolated from various sources of seafood. *Journal of Health Science*, *55*(5), 783-789. https://doi.org/10.1248/jhs.55.783.

Liu, L., Ge, M., Zheng, X., Tao, Z., Zhou, S., & Wang, G. (2016). Investigation of *Vibrio alginolyticus, V. harveyi,* and *V. parahaemolyticus* in large yellow croaker, *Pseudosciaena crocea* (Richardson) reared in Xiangshan Bay, China. *Aquaculture Reports, 3*, 220-224. https://doi.org/10.1016/j.aqrep.2016.04.004.

Marhual, N. P., Das, B. K., Pradhan, J., Swain, P., Mishra, B. K., & Eknath, A. E. (2012). RAPD-PCR and outer membrane protein characterization of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated from diseased shrimp. *Israeli Journal of Aquaculture, 64.*

Mironova, L. V., Afanasev, M. V., Basov, E. A., Urbanovich, L. Y., & Balakhonov, S. V. (2014). A retrospective macro-restrictive analysis of *Vibrio cholerae eltor* strains isolated under epidemic complications in the Russian Far East. *Molecular Genetics, Microbiology and Virology, 29*(2), 77-85. https://doi.org/10.3103/S0891416814020074.

Montieri, S., Suffredini, E., Ciccozzi, M., & Croci, L. (2010). Phylogenetic and evolutionary analysis of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* isolates based on *toxR* gene sequence. *New Microbiologica, 33*(4), 359-372.

Nakazawa, T., Yamada, Y., & Ishibashi, M. (1987). Characterization of hemolysin in extracellular products of *Pseudomonas cepacia*. *Journal of Clinical Microbiology*, *25*(2), 195-198. https://doi.org/10.1128/jcm.25.2.195-198.1987.

Osorio, C. R., & Klose, K. E. (2000). A region of the transmembrane regulatory protein *ToxR* that tethers the transcriptional activation domain to the cytoplasmic membrane displays wide divergence among *Vibrio*

species. *Journal of Bacteriology*, *182*(2), 526-528. https://doi.org/10.1128/jb.182.2.526-528.2000.

Parsons, M. B., Cooper, K. L. F., Kubota, K. A., Puhr, N., Simington, S., Calimlim, P. S., Schoonmaker-Bopp, D., Bopp, C., Swaminathan, B., Gerner-Smidt, P., & Ribot, E. M. (2007). PulseNet USA standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio parahaemolyticus*. *Foodborne Pathogens and Disease*, *4*(3), 285-292. https://doi.org/10.1089/fpd.2007.0089.

Ren, C., Hu, C., Jiang, X., Sun, H., Zhao, Z., Chen, C., & Luo, P. (2013). Distribution and pathogenic relationship of virulence associated genes among *Vibrio alginolyticus* from the mariculture systems. *Molecular and Cellular Probes, 27*(3-4), 164-168. https://doi.org/10.1016/j.mcp.2013.01.004.

Takemura, A. F., Chien, D. M., & Polz, M. F. (2014). Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology, 5*. https://doi.org/10.3389/fmicb.2014.00038.

Takeuchi, H., Shibano, Y., Morihara, K., Fukushima, J., Inami, S., Keil, B., Gilles, A. M., Kawamoto, S., & Okuda, K. (1992). Structural gene and complete amino acid sequence of *Vibrio alginolyticus* collagenase. *Biochemical Journal*, *281*(3), 703-708. https://doi.org/10.1042/bj2810703.

Wang, Q., Liu, Q., Ma, Y., Rui, H., & Zhang, Y. (2007). *LuxO* controls extracellular protease, haemolytic activities and siderophore production in fish pathogen *Vibrio alginolyticus. Journal of Applied Microbiology, 103*(5), 1525-1534. https://doi.org/10.1111/j.1365-2672.2007.03380.x.

Wang, R., Hu, X., Deng, Y., & Gooneratne, R. (2021). Effect of food matrix type on growth characteristics of and hemolysin production by *Vibrio alginolyticus*. *Journal of Food Protection*, 84(8), 1411-1420.

Wayne, P. A. (2017). *CLSI Performance Standards for Antimicrobial Susceptibility Testing*. CLSI Document, Clinical Laboratory Standards Institute (CLSI), Wayne, PA, USA.

Yáñez, R., Bastías, R., Higuera, G., Salgado, O., Katharios, P., Romero, J., Espejo, R., & García, K. (2015). Amplification of *tlh* gene in other *Vibrionaceae* species by specie-specific multiplex PCR of *Vibrio parahaemolyticus. Electronic Journal of Biotechnology*, *18*(6), 459-463. http://dx.doi.org/10.1016/j.ejbt.2015.09.007.

Yang, J., Ju, Z., Yang, Y., Zhao, X., Jiang, Z., & Sun, S. (2019). Serotype, antimicrobial susceptibility and genotype profiles of *Salmonella* isolated from duck farms and a slaughterhouse in Shandong province, China. *BMC Microbiology*, *19*. https://doi.org/10.1186/s12866-019-1570-z.

Yeung, M. & Thorsen, T. (2016). Development of a more sensitive and specific chromogenic agar medium for the detection of *Vibrio parahaemolyticus* and other *Vibrio* species. *Journal of Visualized Experiments*, *117*, e54493. https://doi.org/10.3791/54493.