Polish Journal of Microbiology
2019, Vol. 68, No 4, 429–438
https://doi.org/10.33073/pjm-2019-042

ORIGINAL PAPER

Molecular Identification of *Vibrio alginolyticus* Causing Vibriosis in Shrimp and Its Herbal Remedy

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Submitted 19 April 2019, revised 5 August 2019, accepted 5 August 2019

Abstract

Penaeus monodon is highly susceptible to vibriosis disease. Aims of the study were to identify the pathogen causing vibriosis in *P. monodon* through molecular techniques and develop a biocontrol method of the disease by application of herbal extracts. Shrimp samples were collected aseptically from the infected farm and the bacteria were isolated from the infected region of those samples. Based on phenotypic identification, several isolates were identified as *Vibrio* sp. 16S rRNA gene sequences of the selected isolates exhibited 100% homology with *V. alginolyticus* strain ATCC 17749. An *in vivo* infection challenge test was performed by immersion method with *V. alginolyticus* where these isolates caused high mortality in juvenile shrimp with prominent symptoms of hepatopancreatic necrosis. Antibiogram profile of the isolates was determined against eleven commercial antibiotic discs whereas the isolates were found resistant to multiple antibiotics. A total of twenty-one herbal extracts were screened where *Emblica officinalis*, *Allium sativum*, and *Syzygium aromaticum* strongly inhibited the growth of *V. alginolyticus* in *in vitro* conditions. In *in vivo* conditions, the ethyl acetate extracts of *E. officinalis* and *A. sativum* successfully controlled the vibriosis disease in shrimp at a dose of 10 mg/g feed. This is the first report on molecular identification and biocontrol of *V. alginolyticus* in shrimp in Bangladesh.

Key words: *Penaeus monodon*, hepatopancreatic necrosis, *in vivo* challenge test, *Emblica officinalis*, *Allium sativum*

Introduction

Shrimp culture is one of the fastest-growing aquaculture industries in Bangladesh. A dramatic expansion of shrimp culture occurred in the 1980s significantly contributed to the economy of Bangladesh (Paul and Vogl 2011; Hossain et al. 2013). However, in the recent years, shrimp production in Bangladesh has severely been affected by the outbreak of various diseases such as black spot, softshell, external fouling, broken appendages, hepatopancreatic infection, and vibriosis (Chowdhury et al. 2015; Ali et al. 2018). Among these diseases, vibriosis is considered as one of the most important bacterial diseases in shrimp farms of Bangladesh. Vibriosis infects both penaeids and non-penaeid shrimps (Chowdhury et al. 2015) and is responsible for high mortality in aquaculture worldwide (Lightner 1988; Sparagano 2002), and can devastate the entire shrimp farm (Wei and Wendy 2012). Several members of *Vibrio* genus such as *V. harveyi*, *V. anguillarum*, *V. splendidus*, *V. parahaemolyticus*, *V. fluvialis*, and *V. alginolyticus* are reported as the causative agents of vibriosis in shrimp (de la Pena et al. 1993; Karunasagar et al. 1994; Lee et al. 1996; Lightner 1996; Austin and Zhang 2006; Chatterjee and Halder 2012). No molecular level study has so far been conducted to precisely identify the causal agent of vibriosis in shrimp in Bangladesh and the required effective management for this worrisome disease.

A number of antibiotics and chemotherapeutic agents have been used in shrimp farms to prevent and

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control of microbial diseases including vibriosis (Mohney et al. 1992; Hossain et al. 2012; Karim et al. 2018). But indiscriminate and careless use of antibiotics leads to the development of antibiotic resistance in microorganisms (Karunasagar et al. 1994), which is now a major health concern worldwide (Karim et al. 2018). Therefore, an alternative approach is needed for effective and sustainable management of vibriosis in shrimp. Herbal extracts could be used as safe and alternative to synthetic antibiotics for the management of vibriosis in shrimp. Although *Vibrio* sp. is the important causal pathogen in shrimp farms, the investigation of the herbal remedy of vibriosis in shrimp is very limited in Bangladesh. Bangladesh is rich in diversed medicinal plants (Yusuf et al. 2009) and some of them have been found effective in the management of fish diseases. It has been demonstrated that methanol extracts of *A. sativum,* and methanol and acetone extracts of *S. aromaticum* significantly reduce the mortality of tilapia fish, artificially infected with *Enterococcus faecalis* as both preventive and therapeutic agents (Sindermann 1990). However, no study has so far been conducted in Bangladesh for the management of shrimp diseases caused by *Vibrio* sp. using herbal extracts. Therefore, the objectives of this study were to (i) identify the causal agent(s) of vibriosis in shrimp through physiological, biochemical and molecular techniques, (ii) assess the antibiotic susceptibility profile in the isolated shrimp pathogenic *Vibrio* sp., and (iii) control of vibriosis through the treatment with herbal extracts.

Experimental

Materials and Methods

Isolation and phenotypic identification of bacteria from the infected shrimp. Shrimp (*P. monodon*) suspected to be suffering from vibriosis were collected from different farms located in Satkhira district (near to Sundarbans mangrove forest at the south-west part of Bangladesh). The moribund shrimp were collected and individually kept in sterilized polythene bags and transported to the laboratory of the Faculty of Fisheries of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh maintaining proper icing and aseptic condition. The symptoms of the infected shrimp such as deformed and discolored (blackish or yellowish) hepatopancreas and reddening of the body (Table I) were recorded. The hepatopancreas of the shrimps were dissected and the samples were serially diluted. Hundred microliters of the diluted (10^{-5} to 10^{-7}) samples were spread on nutrient agar (NA; supplemented with 2% NaCl) and thiosulfate citrate bile salt (TCBS) agar plates to isolate the bacteria (Shaanmugasundaram et al. 2015). The agar plates were incubated at 28°C for 24–48 hours in an incubator (Liu et al. 2004). Several colonies were randomly selected from each plate and inoculated on NA media to obtain a pure culture. The isolates were routinely sub-cultured on NA plates and stock cultures were maintained in nutrient broth supplemented with 2% NaCl and 10% glycerol and stored in a freezer at –20°C. Individual colonies grown on NA or TCBS plates were observed and colony characteristics such as colony size, shape, color, type, etc. were recorded. To identify the isolates, Gram's staining, bacterial shape, motility, catalase, oxidase, oxidative-fermentative (O-F) test, acetoin production, indole production, arginine dihydrolase, lysine decarboxylase, hydrogen sulfide (H_2S) production, acid production from glucose, arabinose, mannitol, sorbitol and sucrose, sensitivity to Vibriostatic agent 0/129, growth at 4°C and 40°C were observed (Alsina and Blanch 1994; Rahman et al. 2010). Growth of the bacterial isolates in different salt concentrations was studied by supplementing the NA media with 2, 4, 6, and 8% of NaCl and incubated at 28°C. Growth in the absence of NaCl was studied by removing the NaCl from the NA.

Molecular identification of *Vibrio* isolates. Of a total of 20 isolates, genomic DNA of four representatives of *Vibrio* isolates (2A1a, 2A3, 2A11 and 2V21) was extracted using a commercial DNA extraction kit (GeneJET Genomic DNA purification Kit K0721, Thermo Scientific). The quantity of the extracted DNA was checked by electrophoresis on 0.8% agarose gel and compared with a lambda DNA marker (Promega). The DNA was stored at –20°C for further use. Polymerase chain reaction (PCR) for amplification of the targeted

Table I
External symptoms of vibriosis and the site of isolation of pathogen from infected shrimp collected from the shrimp farms.

Sample No.	Symptoms	Site of isolation
01–04	Deformed and yellowish colored hepatopancreas	Hepatopancreas
05–06	Blackish colored hepatopancreas	Hepatopancreas
07–11	Discolored hepatopancreas	Hepatopancreas
12–13	Yellowish colored hepatopancreas	Hepatopancreas
14–15	No visible symptom	Hepatopancreas

16S rRNA gene sequences of the isolates was performed with universal primer sets 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Sigma Ltd.). Each PCR mixture contained 6 µl of 25 mM $MgCl_2$, 10 µl of 10 × PCR buffer, 2.0 µl of 10 mM deoxyribonucleotide triphosphate, 5.0 µl of a 20 µM solution of each primers, 100–200 ng of DNA template, 0.5 µl of *Taq* DNA polymerase (Promega) at 5 U µl/l, and sterile double-distilled water in a total volume of 100 µl. The PCR amplification was performed in a PCR thermocycler (Eppendorf Ltd.). The optimal conditions for PCR were set as follows: an initial denaturation step at 94°C for 5 min; 35 cycles of a denaturation step at 94°C for 1 min, an annealing at 57°C for 40 second, and an extension at 72°C for 1 min and a final extension step at 72°C for 7 min. A small portion (usually 5 µl) of the PCR amplicons were mixed with 1–2 µl of 6 × loading dye and loaded in a 1.5% agarose gel with 1 Kb ladder marker (Promega). Then, electrophoresis was performed in 0.5 × Tris-Borate-EDTA (TBE) buffer for 40 min at 70 volts. Amplicons were visualized with UV light in a gel documentation system (Weltec KETA G, Weltec Corp.). The PCR product was purified using a commercial Gel/PCR Purification Kit (FavorPrep TM, Favorgen® Biotech Corp.) following the manufacturer's protocol. The DNA sequencing was done in the Center for Advanced Research in Sciences (CARS) at the University of Dhaka in a DNA sequencer (ABI-3130, Applied BioSystems). The sequence data was extracted by using BIOAD software as FASTA format. The DNA sequences (FASTA format) of the isolates were then analyzed using web-based Basic Local Alignment Search Tool (BLAST) program of National Centre for Biotechnology Information (NCBI) and phylogenetic analysis was done using the Phylogeny.fr web-based software.

***In vivo* challenge test.** To evaluate the pathogenic potential of the isolates, four representatives of *Vibrio* sp. isolates (2A1a, 2A3, 2A11 and 2V21) were used for *in vivo* challenge test following the immersion method with three replications. We followed Completely Randomized Design (CRD) for the experiment. Juvenile shrimp (average length 3.5 ± 0.13 cm, weight 2.1 ± 0.4 g) were collected from a nursery pond of a private hatchery and acclimatized in an aquarium at room temperature and continued for seven days. Bacterial inoculums for infection challenge test were prepared by adding 30 ml of 24 hours TCBS broth culture into 2970 ml sterile saline solution (2% w/v NaCl). TCBS broth culture was used for specific and better growth of *Vibrio* bacterium. The density of the inoculums was 4.2×10^6, 3.8×10^6, 5.6×10^6, and 5.4×10^6 CFU/ml for the isolates 2A1a, 2A3, 2A11, and 2V21, respectively. Sixty juvenile shrimp (twenty juveniles in each aquarium) were immersed into individual bacterial suspension for 20 minutes at room temperature (about 25°C) and then transferred to three separate aquaria containing 15 l saline water. A group of shrimp was maintained as a negative control, which was not inoculated with any bacterial suspension. The treated juveniles were regularly monitored at six hours interval for seven days, and the signs of infection and/or mortality was recorded. The mortality data were analyzed by ANOVA at $< 0.05 >$ level of significance. Bacteria were re-isolated from the hepatopancreases of dead and infected shrimps on NA agar supplemented with 2% NaCl and identified based on their phenotypic characteristics (Rahman et al. 2017).

***In vitro* antibiogram assay.** Susceptibility profile of four representatives of *Vibrio* isolates (2A1a, 2A3, 2A11 and 2V21) was determined by disc diffusion method (Jorgensen and Ferraro 2009; Rahman and Hossain 2010) against of eleven commercial antibiotic discs. Antibiotic discs used in this study were erythromycin (15 µg/disc), penicillin (10 µg/disc), cefradine (25 µg/disc), levofloxacin (5 µg/disc), azithromycin (30 µg/disc), amoxicillin (30 µg/disc), cefuroxime (30 µg/disc), vancomycin (30 µg/disc), nitrofurantoin (30 µg/disc), ampicillin (25 µg/disc), and gentamycin (10 µg/disc) (manufactured by HiMedia Laboratories Pvt. Ltd.). Bacterial culture was spread on the Isosensei test agar plates (Traub et al. 1998; BSAC 2015; Rahman et al. 2017) and the antibiotic discs were placed on the culture plates and incubated at 37°C for 24 hours in an incubator. After incubation, the diameter of the inhibition zone (if any) was measured. The isolates were considered as sensitive or resistant according to CLSI-specified interpretive criteria (CLSI 2005).

***In vitro* inhibitory activity of herbal extracts.** Twenty-one medicinal herb extracts were used in this study to screen their inhibitory activity against the *Vibrio* isolates (Table II). The plants were selected based on their recognized medicinal properties described elsewhere (Muniruzzaman and Chowdhury 2004; Rahman and Hossain 2010; Rahman et al. 2017). For the preparation of herb extracts, 25 g of dried fresh herb materials were added into 100 ml sterile distilled water, *n*-hexane, ethyl acetate, acetone, and methanol. The samples were gently rotated in an orbital shaker at room temperature for 72 hours and then evaporated in a rotary evaporator at 50°C. The dried extracts were dissolved in respective solvents to adjust the concentration at 25 mg/ml. Sterilized filter paper discs were soaked with 30 µl (25 mg/ml solvent) of aqueous, *n*-hexane, ethyl acetate, acetone, and methanol extracts and their inhibitory activities were determined as described elsewhere (Rahman et al. 2017). All plates were incubated at 37°C for 24 hours and the diameter of the discs and the zone of inhibition were measured, and ratios between the diameters were calculated (Rahman et al. 2017). Data were collected from three replicated plates for each plant extract and calculated the mean value with standard deviation.

Table II
Medicinal herbs used for *in vitro* antibacterial assay against *V. alginolyticus* isolates.

Sl. No.	English name	Scientific name	Plant parts used
1	Zinger	*Zingiber officinale*	Rhizome
2	Turmeric	*Curcuma longa*	Rhizome
3	Clove	*Syzygium aromaticum*	Bud
4	Garlic	*Allium sativum*	Bulb
5	Onion	*Allium cepa*	Bulb
6	Black cumin	*Nigella sativa*	Seed
7	Mehogoni	*Swietenia mahagoni*	Seed
8	Bottle gourd	*Laganaria siceraria*	Seed, Fruit
9	Guava	*Psidium guajava*	Fruit
10	Olive	*Olea europaea*	Fruit
11	Chilli	*Capsicum pendulum*	Fruit
12	Rose periwinkle	*Catharanthus roseus*	Leaf and flower
13	Amla	*Emblica officinalis*	Leaf
14	Tamarind	*Tamarindus indica*	Leaf
15	Arjun	*Terminalia arjuna*	Leaf
16	Papaya	*Carica papaya*	Leaf
17	Carunda	*Carissa carandas*	Leaf
18	Bermuda grass	*Cynodon dactylon*	Leaf
19	Neem	*Azadiracha indica*	Leaf
20	Pomegranate	*Punica granatum*	Leaf
21	Carambola	*Averrhoa carambola*	Leaf

***In vivo* control of vibriosis infection by application of herbal extracts.** *In vivo* effects of ethyl acetate extract of *E. officinalis* leaf, ethyl acetate, and methanol extracts of *A. sativum* bulb, methanol, and acetone extracts of *S. aromaticum* bud, and acetone extract of *T. indica* leaf used as therapeutic agents against vibriosis infection in shrimp were evaluated in laboratory conditions. For this purpose, the stock solutions of 25 mg/ml ethyl acetate extracts of *E. officinalis*, ethyl acetate and methanol extract of *A. sativum*, methanol and acetone extract of *S. aromaticum* and acetone extract of *T. indica* were prepared. The herbal extracts were mixed with a commercial feed for juvenile shrimp at the dose of 5, 10, and 15 mg extract/g feed (0.2, 0.4, 0.6 ml from stock solution). Juvenile shrimp were exposed with 24 h culture suspension of a representative *V. alginolyticus* isolate (2A1a) as described earlier. The different groups of *V. alginolyticus* exposed shrimp, each of which had 3 replicates (n = 20), were transferred to different aquarium. A group of juvenile shrimp exposed to the bacterial suspension and fed with normal commercial feed (without any plant extract) was described as control group-2. Another group of shrimp not exposed to the bacterial suspension and fed with normal commercial feed (without any herb extract) was known as control group-1. The rest groups of juvenile shrimp exposed to bacterial suspension were fed with commercial feed supplemented with ethyl acetate extracts of *E. officinalis*, ethyl acetate and methanol extract of *A. sativum*, methanol and acetone extract of *S. aromaticum* and acetone extract of *T. indica* at different doses (5, 10, and 15 mg extract/g feed). The juvenile shrimp were supplied feed at a rate of 10% of their body weight. Shrimp were fed twice in a day at 12 hours interval. Continuous aeration was maintained in the aquarium and approximately 50% of water was exchanged in two days interval. The experiment was continued for seven days.

Statistical analysis of data. Experiments for *in vivo* challenge test and *in vivo* control of the vibriosis infection by application of herbal extracts were carried out using a complete randomized design (CRD). Data were analyzed by one-way analysis of variance (ANOVA) and the mean values were separated by LSD posthoc statistic. The level of significance was $p < 0.05$. All the analyses were performed using Statistics 10. Mean value ± standard error of 3 replications was used in Tables and Figures.

Results

Isolation, phenotypic, and molecular characterization of the pathogen causing vibriosis in shrimp. The infected shrimps were collected from the farms near Sundarbans mangrove forest**.** The high mortality of shrimps (approximately 65%) was observed in these farms. The infected shrimps exhibited reduced feeding and lethargic in swimming at the edges and surface of the water body. No symptoms of white spot syndrome virus disease (WSSV) were observed in the infected farms. The farms were also free from pollution. Water quality parameters of the infected farms were 7.8 ± 0.2, 30 ± 1.6, 8.6 ± 1.0, 0.2 ± 0.08, and 4.5 ± 0.5 for pH, temperature (°C), salinity (ppt), ammonia (mg/l), and dissolved oxygen (mg/l), respectively. Twenty-five bacterial isolates were randomly selected (18 from TCBS and 7 from NA) as suspected *Vibrio* sp. based on their colony characteristics for preliminary phenotypic identification. Among these, a total of 20 isolates (16 from TCBS and 4 from NA) were Gram-negative, rod-shaped, fermentative, motile bacteria, susceptible to vibriostatic agent 0/129, and were positive in oxidase test, catalase test, indole production, and lysine decarboxylase test but negative in acetoin and hydrogen sulfide (H_2S) production test (Table III). Isolates were able to grow in the presence of 2, 4, 6, and 8% NaCl but did not grow in the absence of NaCl. They were unable to grow at 4°C but grew well at 40°C temperature. The isolates produced acid from glucose, sorbitol, mannitol, and sucrose but did not produce acid from arabinose. Based on the

Table III
Colony, morphological, and biochemical characteristics of *Vibrio* sp. isolates.

Test Type	Test	Characteristics
Colony characteristics	Color in NA media	Brownish
	Color in TCBS media	Yellowish
	Size	Large
	Shape	Round
	Elevation	Convex
Morphological characteristics	Shape	Comma
	Motility	+
	Growth in 0% NaCl	–
	Growth in 2,4 and 8% NaCl containing media	+
	Growth at 4°C	–
	Growth at 40°C	+
Biochemical characteristics	Gram's staining	–
	Oxidative-Fermentative	F
	Oxidase	+
	Catalase	+
	Acetoin production	–
	H_2S production	–
	Indole	+
	Sensitivity to a vibriostatic agent 0/129	+
	Arginine dihydrolase	–
	Lysine decarboxylase	+
Acid production from	Glucose	+
	Arabinose	–
	Manitol	+
	Sorbitol	+
	Sucrose	+

Note: + = Positive reaction; – = Negative reaction; F = Fermentative

colony's morphological and biochemical characteristics, twenty isolates were phenotypically identified as *Vibrio* sp. (Farmer et al. 2005; Jayasree et al. 2006; Nelapati et al. 2012). The phenotypic and biochemical characteristics of these twenty isolates were very similar to the characteristics described for *V. alginolyticus* (Lie et al. 2004).

Among twenty isolates, four (2A1a, 2A3, 2A11 and 2V21) were randomly selected for further molecular, pathological, antibiotic susceptibility and herbal disease control studies. The 16S rRNA gene sequence data of these four selected isolates exhibited 100% homology with *V. alginolyticus* strain ATCC 17749. The sequences of the isolates 2A1a, 2A3, 2A11, and 2V21 have been deposited to NCBI Gen Bank with accession numbers MG757701, MG757699, MG757700, and MG757703, respectively. In the phylogenetic tree, these four isolates shared a common ancestor and formed a cluster with *V. alginolyticus* (Fig. 1).

***In vivo* challenge of the isolated *V. alginolyticus*.** To observe whether *V. alginolyticus* isolates were pathogenic to juvenile shrimp, we conducted an *in vivo* challenge test under laboratory conditions. All of the four *V. alginolyticus* isolates tested (2A1a, 2A3, 2A11, and 2V21) produced disease symptoms in the juvenile shrimp and caused high mortality ranged from 81.67 ± 2.29% to 86.67 ± 2.29% (Fig. 2). In juvenile shrimp, mortality was observed from 24 to 96 hours after inoculation and the highest mortality was recorded within 72 hours. Hepatopancreatic discoloration, the main symptom of infection, was observed clearly in the challenged shrimp within 96–144 hours. The infected shrimp exhibited feeding redundancy and lethargic swimming at the surface of the aquarium.

Antibiogram profile of *V. alginolyticus* isolates. To find out whether the shrimp pathogenic *V. alginolyticus* isolates had any resistance against commercial antibiotics, we screened them against eleven antibiotics using disc diffusion assay. *V. alginolyticus* isolates exhibited resistance against various antibiotics such as erythromycin, penicillin, amoxicillin, vancomycin, ampicillin, and cefradine (Table IV). However, they were found sensitive to levofloxacin, cefuroxime, azithromycin,

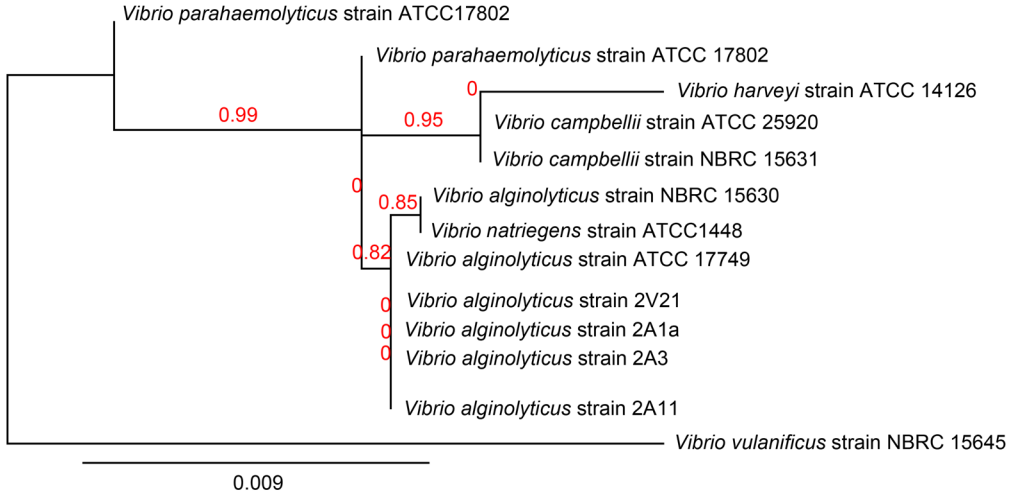

Fig. 1. Unrooted phylogenetic tree showing evolutionary relationship of *V. alginolyticus* isolates with other maximum identical related species on the basis of 16S rRNA gene sequences evolutionary distance.

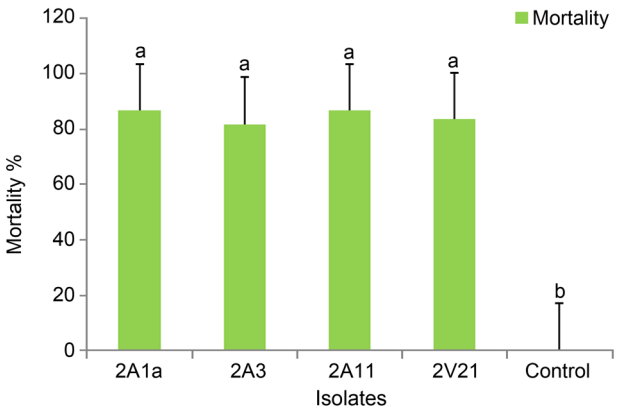

Fig. 2. Mortality of juvenile shrimp exposed to *V. alginolyticus* isolates in laboratory conditions. One way ANOVA was performed at ≤0.05 level of significance. Same letters indicate there is no significant variations in mortality of shrimp in different groups challenged with different isolates of *V. alginolyticus*.

nitrofurantoin, and gentamicin. The maximum and minimum inhibition zone was observed for levofloxacin and azithromycin, respectively.

***In vitro* inhibitory effects of herbal extracts.** The shrimp pathogenic *V. alginolyticus* isolates were susceptible to crude aqueous extracts of the leaf of *E. officinalis*, the bulb of *A. sativum*, the bud of *S. aromaticum*, and the leaf of *T. indica*. The *E. officinalis* extracts displayed the highest antibacterial activity (Table V). The bulb extract of *A. sativum* and bud extract of *S. aromaticum* also showed high inhibitory activity against *V. alginolyticus* whereas, lowest antibacterial activity against the *V. alginolyticus* isolates was observed for the leaf extract of *T. indica*. The ethyl acetate extract of the *E. officinalis* leaf strongly inhibited the growth of *V. alginolyticus* with maximum zone ratio 6.4 ± 0.19 but methanol, *n*-hexane, and acetone extracts of the leaf of *E. officinalis* caused no inhibition. The ethyl acetate extract of the bulb of *A. sativum* also highly inhibited *V. alginolyticus* (Fig. 3) followed by methanol extract. Acetone and methanol extracts of the bud of *S. aromaticum* also strongly inhibited the growth of *V. alginolyticus* isolates. The acetone, *n*-hexane, methanol, and ethyl acetate extracts of the leaf of *T. indica* inhibited the growth of *V. alginolyticus* but the zone ratios were not satisfactory.

***An in vivo effect of herbal extracts* as therapeutic agents against vibriosis in shrimps.** Juvenile shrimps were fed with various herbal extracts mixed feed after

Table IV
Iv vitro antibiogram profiles of the *V. alginolyticus* isolates. Eleven commercial antibiotic discs were used.

Isolates	Inhibition zone ratio against different antibiotics										
	Er	Pe	Am	Va	Amp	Le	Cx	Az	Ni	Ce	Ge
2A1a	R	R	R	R	R	7.3 ± 0.2	4.6 ± 0.3	3.8 ± 0.3	6.8 ± 0.1	R	6 ± 0.2
2A3	R	R	R	R	R	7.3 ± 0.3	3.7 ± 0.2	3.3 ± 0.1	7 ± 0.1	R	5.7 ± 0.1
2A11	R	R	R	R	R	7.2 ± 0.1	4.8 ± 0.6	3.7 ± 0.6	6.5 ± 0.5	R	5.8 ± 0.1
2V21	R	R	R	R	R	7.0 ± 0.1	4.2 ± 0.2	3.3 ± 0.2	7.0 ± 0.2	R	5.7 ± 0.3

Note: Er = Erythromycin (15 µg/disc), Pe = Penicillin (10 µg/disc), Am = Amoxycillin (30 µg/disc), Va = Vancomycin (30 µg/disc), Amp = Ampicillin (25 µg/disc), Le = Levoflaxin (5 µg/disc), Cx = Cefuroxime (30 µg/disc), Az = Azithromycin (30 µg/disc), Ni = Nitrofurantoin (30 µg/disc), Ce = Cefradine (25 µg/disc), Ge = Gentamicin (10 µg/disc), R = Resistant.

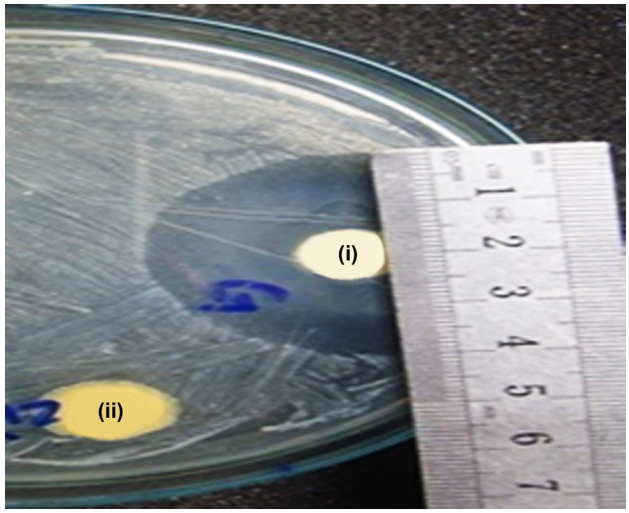

Fig. 3. An *in vitro* antibacterial activity of disc containing organic solvent extracts of herbs. (i) EtOAc extract of *A. sativum,* and (ii) control (no plant extract).

exposing them to the highly virulent isolate of *V. alginolyticus* (2A1a). Interestingly, 100 ± 0.0% of the challenged shrimp were survived when fed with ethyl acetate extract of *E. officinalis* and *A. sativum* at the rate of 10 mg/g of feed (Fig. 4). Survival of shrimp was also high (86.7 ± 5.8%) when fed with methanol extract of *A. sativum* at the rate of 10 mg/g of feed. Hoverer, only 16.7 ± 2.87% shrimp survived when challenged with the pathogenic *Vibrio* isolate 2A1a in control group 2 (normal feed without any herbal treatment). The lower rate of survival (35.0 ± 7.07%) was also observed in the shrimp when fed with acetone extract of *T. indica* at a rate of 15 mg/g feed.

Discussion

Vibriosis is one of the most important bacterial diseases of shrimp caused by several species of *Vibrio*. This study isolated and identified (phenotypically) twenty isolates of *Vibrio* sp. from the infected shrimp with vibriosis symptoms collected from shrimp farms of Bangladesh. Among twenty, four virulent isolates were identified as *V. alginolyticus* by 16S rRNA gene sequencing (Fig. 1). These *V. alginolyticus* isolates exhibited a high level of virulence against juvenile shrimp (81.67 ± 2.29% to 86.67 ± 2.29% mortality) in an *in vivo* challenged study. Liu et al. (2004) also obtained 80% mortality in shrimp in an *in vivo* challenge test

Table V
An *in vitro* inhibitory activity of herbal extracts on shrimp pathogenic *V. alginolyticus* isolates.

Plants	Type of extracts	Inhibition zone ratio of herbal extracts for *V. alginolyticus* isolates			
		2A1a	2A3	2A11	2V21
E. officinalis	Aqueous extract	5.33 ± 0.64	4.17 ± 0.38	4.67 ± 0.12	4.10 ± 0.44
	n-Hexane extract	–	–	–	–
	EtOAc extract	6.1 ± 0.19	5.6 ± 0.20	5.0 ± 0.23	6.1 ± 0.07
	MeOH extract	–	–	–	–
	Acetone extract	–	–	–	–
A. sativum	Aqueous extract	4.00 ± 0.46	4.10 ± 0.10	4.60 ± 0.53	3.80 ± 0.66
	n-Hexane extract	1.9 ± 0.06	1.8 ± 0.15	1.6 ± 0.05	1.8 ± 0.17
	EtOAc extract	4.1 ± 0.11	4.3 ± 0.03	3.3 ± 0.05	3.8 ± 0.25
	MeOH extract	2.5 ± 0.06	2.1 ± 0.25	2.4 ± 0.11	1.9 ± 0.15
	Acetone extract	1.5 ± 0.25	1.4 ± 0.15	1.4 ± 0.36	1.5 ± 0.06
S. aromaticum	Aqueous extract	3.93 ± 0.15	3.47 ± 0.55	3.93 ± 0.21	3.50 ± 0.53
	n-Hexane extract	3.5 ± 0.04	3.6 ± 0.24	3.6 ± 0.13	3.8 ± 0.14
	EtOAc extract	–	–	–	–
	MeOH extract	4.9 ± 0.21	4.6 ± .17	4.0 ± 0.06	4.4 ± 0.08
	Acetone extract	4.3 ± 0.12	4.4 ± 0.06	4.0 ± 0.15	4.1 ± 0.22
T. indica	Aqueous extract	1.17 ± 0.40	0.97 ± 0.21	1.20 ± 0.36	0.93 ± 0.15
	n-Hexane extract	1.9 ± 0.09	1.8 ± 0.06	1.5 ± 0.09	1.8 ± 0.21
	EtOAc extract	1.8 ± 0.14	1.9 ± 0.21	1.9 ± 0.06	1.5 ± 0.08
	MeOH extract	1.5 ± .22	1.5 ± .19	1.4 ± 0.08	1.4 ± 0.11
	Acetone extract	2.3 ± 0.12	1.8 ± 0.11	1.5 ± 0.13	1.8 ± 0.05

Note: Eight millimeter diameter filter paper discs were soaked with 30 microliter of aqueous, *n*-hexane, ethyl acetate (EtOAc), methanol (MeOH) and acetone extracts (25 mg/ml) of *E. ofiicinalis*, *A. sativum*, *S. aromaticum*, and *T. indica* and then allowed to dry in a laminar airflow cabinet before placing them to the NBA petri dish inoculated with respective isolates of the pathogen. Each treatment was replicated for three times. Data presented here is the mean ± SE.

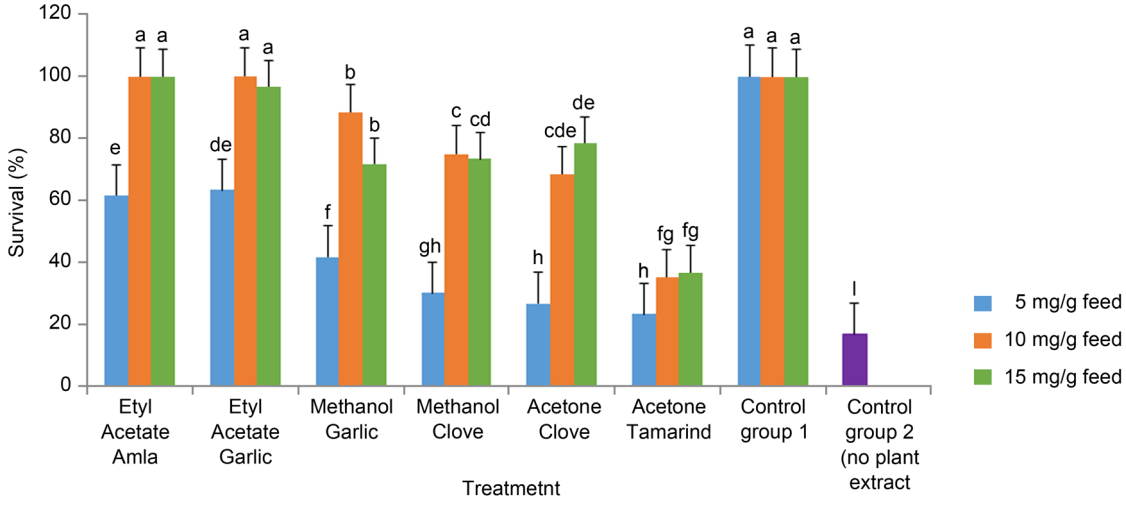

Fig. 4. Survival rate of shrimps fed with herbal extracts at day 7 after infection with a virulent strain of *V. alginolyticus* 2A1a. One way ANOVA was used for analysis of the data and mean value with standard deviation in the bar followed by the same letter (s) are not significantly different as assessed by LSD (Least Significance Difference) at $p \leq 0.05$. Control group-1 was not exposed to the pathogen; Control group-2 was infected with the pathogen but fed normal commercial feed.

with *V. alginolyticus* (CH003). In this study, the infected shrimp in *in vivo* challenge test exhibited almost identical symptoms as found in the naturally infected shrimp (Lightner 1993; Anderson et al. 1998).

One of the important findings of this study is that the shrimp pathogenic *V. alginolyticus* isolates showed resistance against various antibiotics but exhibited the high level of susceptibility to both aqueous and organic solvent extracts of herbs such as *E. officinalis*, *A. sativum*, *S. aromaticum*, and *T. indica* (Table V). Among these, ethyl acetate extract of *E. officinalis* strongly inhibited the growth of *V. alginolyticus* strains followed by ethyl acetate and methanol extract of *A. sativum,* and methanol extract of *S. aromaticum*. However, lower inhibition was obtained for both aqueous and organic extracts of *T. indica*. Medicinal plants are used to treat a variety of diseases for thousands of years of civilizations (Petrovska 2012). An *in vitro* antibacterial activity of numerous plants against both Gram-positive and Gram-negative marine bacteria has been reported elsewhere (Castro et al. 2008; Roomiani et al. 2013). Ethanol extract of turmeric (*Curcuma longa*) was reported to inhibit the shrimp pathogen *Vibrio* sp. (Lawhavinit et al. 2011). *In vitro* inhibitory activities of both aqueous and organic solvent extracts of *A. sativum*, *S. aromaticum,* and *T. indica* against fish pathogenic *Enterococcus faecalis* have also been reported (Rahman et al. 2017).

The most remarkable finding of this study is that ethyl acetate extracts of *E. officinalis* and *A. sativum* remarkably protected the juvenile shrimp (up to 100%) from vibriosis by a virulent isolate of *V. alginolyticus* (Fig. 4). High to moderate survival rates were also obtained in shrimp fed with methanol extracts of *A. sativum* and *S. aromaticum*, and acetone extract of *S. aromaticum*. Inhibition of *V. alginolyticus* both in *in vitro* and *in vivo* conditions by organic solvent extracts of *E. officinalis*, *A. sativum,* and *S. aromaticum* suggests that these herbal extracts contain antibacterial secondary metabolite(s). Medicinal herbs are considered as one of the most important sources for medicine and drugs, as many secondary metabolites including antimicrobial substances are obtained from various herbs. Plants possess complex chemicals with varied biological activities, making plants suitable for the treatment of multifactorial diseases, and makes plants a suitable alternative to antibiotics with little risk for development of resistance (Gostner et al. 2012; Srivastava et al. 2014). *A. sativum* contains several bioactive compounds such as ajone, allicin and diallyl sulfides that possess potential antibacterial activity against different microorganisms (Naganawa et al. 1996; Ankri and Mirelman 1999; O'Gara et al. 2000). *S. aromaticum* contains eugenol that is reported to exhibit strong antibacterial activity against *Staphylococcus aureus* (Xu et al. 2016). Cinnamaldehyde and its derivatives obtained from cinnamon were reported to reduce the virulence in *Vibrio* sp. causing vibriosis (Brackman et al. 2008). *S. cumini* leaf powder also reported to increase immunity in juvenile shrimp (*Litopenaeus vannamei*) against *V. parahaemolyticus* infection (Prabu et al. 2018). Organic solvent extracts of *A. sativum* and *S. aromaticum* also reported to significantly increase the survival of *Oreochromis niloticus* from infection against *E. faecalis* (Rahman et al. 2017). Herbal extracts are also reported to stimulate immunity and develop disease resistance in shrimp (Raja Rajeswari et al. 2012; Yogeeswaran et al. 2012). Dietary administration of *Gynura bicolor* extract was reported to enhance the innate immunity and antio-

xidant enzyme activities of shrimp against *V. alginolyticus* and WSSV infection (Wu et al. 2015). The purified garlic compounds allicin and ajoene demonstrated immune stimulant capacity against fish pathogenic protozoa *Spironucleus vortens* and *Ichthyophthirius multifiliis*, and the bacteria *A. hydrophila* (Nya et al. 2010; Tanekhy and Fall 2016). Recently, Foysal et al. (2019) reported that dietary administration of garlic could modulate gut microbiota, increase recovery from streptococcus infection and upregulate the expression of immune genes in the intestinal tissue of tilapia. Since crude plant extracts contain multiple secondary metabolites, the chances of development of resistance in the pathogens against these extracts are likely lesser than those of pure antibiotics (Rahman et al. 2017). Valuable drugs could be developed form these herbal extracts to control vibriosis in shrimp and other fish diseases. The extracts of *E. officinalis* and *A. sativum* could be used as alternative therapeutic agents against vibriosis disease in shrimp.

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Acknowledgements

The authors acknowledge the Ministry of Science and Technology, Government of Bangladesh for providing special allocation under a research project titled "Molecular Detection and Bio-control of the Pathogen Causing Vibriosis and Black Spot Diseases in Prawn and Shrimp" to conduct the research work. Thanks to Mr. Nabangshu Shekhar Das of the University of Calgary, Canada for English language correction.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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