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Isolation and identification of *Vibrio nereis* and *Vibrio harveyi* in farm raised *Penaeus monodon* marine shrimp

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Abstract

The present research work was conducted for the isolation and identification of *Vibrio nereis* and *Vibrio harveyi* in farm raised *Penaeus monodon* shrimp on three commercial ghers. Shrimp (n=6) were collected from three ghers located at Satkhira district of Bangladesh. Intestinal (n= 6) samples were collected and the intestine of shrimp was taken into a test tube containing 10 ml of sterile distilled water and mixed well by vortex mixer machine. The resulting solution was then used to prepare serial dilution. 1ml of this suspension was transferred to 9 ml of sterile distilled water for tenfold (1:10) dilution and further diluted up to 10^4 dilutions. For enumeration of bacteria 1ml of diluted samples were inoculated on petri plate aseptically before pouring the nutrient agar on the plates and incubated at 10°C, 27°C, 37°C and 45°C for 24-48 hours. After incubation total bacteria was counted and well-spaced colony was marked for isolation. Isolated colony was then streaked on nutrient agar for pure culture. For isolation of *Vibrio* spp. pure bacterial culture was then streaked on TCBS agar plate. Identification of bacteria was performed by cultural, staining and biochemical properties. One *Vibrio harveyi* and one *Vibrio nereis* isolates were identified in *Penaeus monodon* shrimp. The results of this study indicate that *Penaeus monodon* shrimp harbor *Vibrio harveyi* and *Vibrio nereis* which might cause vibriosis in shrimp and public health problem if enter into human food chain.

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Introduction

The genus Vibrio includes Gram-negative, oxidase-positive (except two species), rod- or curved rod-shaped facultative anaerobes (FDA, 1992). Five types of diseases such as: tail necrosis, shell disease, red disease, loose shell syndrome (LSS) and white gut disease (WGD) is caused by Vibrio spp. in Penaeus monodon (Jayasree *et al.*, 2006). Many Vibrio species are pathogens to human and have been implicated in food borne disease (FDA, 1992).

Few reports are available on the isolation and identification of *V. harveyi and V. nereis* from farm raised marine shrimp in Bangladesh. (Shafiqur Rahman *et al.*, 2010). To the best of our knowledge no study has been conducted on the isolation and identification of *V. harveyi and V. nereis* in farm raised *Penaeus monodon* shrimp at ghers in the Satkhira districts of Bangladesh. The objectives of this study were (i) Isolation of *Vibrio harveyi* and *Vibrio nereis* from farm raised *Penaeus monodon* shrimp and (ii) Identification of *Vibrio harveyi* and *Vibrio nereis* from farm raised *Penaeus monodon* shrimp collected from Satkhira district of Bangladesh.

Materials and methods

Collection of sample

A total of 6 shrimps were collected from three ghers which were located at Satkhira (n=2, Satkhira Sadar and Assasuni Upazilla) district of Bangladesh in the period of January to June, 2014. The samples were placed into the sterile polyethene bags and transported to the Department of Microbiology at the Jessore Science and Technology University (JSTU), Jessore using an ice box aseptically for bacteriological analysis.

Processing of sample

The intestine of shrimp was taken into a test tube containing 10 ml of sterile distilled water and mixed well by vortex mixer machine. The resulting solution was then used to prepare serial dilution. One ml of this suspension was transferred to 9 ml of sterile distilled water for tenfold (1:10) dilution and further diluted up to 10^4 dilutions.

Enumeration of bacteria

For the enumeration and isolation of bacteria, serial dilution was carried out (Chesebrough, 2000). For this purpose 1 ml of each diluted samples were inoculated on sterilized petri dish by using a sterile pipette and 15-20mlof melted Nutrient Agar Media was poured on the petri dishes. Media plates were incubated at 10°C, 27°C, 37°C and 45°C for 24 to 48 hours for enumeration of bacterial colony. After 1 to 2 days of incubation, the plates having well-spaced colonies of different bacteria were selected for counting. The selected plates were placed on a colony counter and the colonies were counted precisely by naked eye. The counts of colonies were considered as per ml and were calculated by multiplying the average number of colonies per plate by the reciprocal of the dilution factor. The calculated results were expressed as colony forming units (cfu) per ml of sample.

Isolation of bacteria

After enumeration of the plates, highly well-spaced colonies were selected for isolation. The selected colonies were marked and their morphological (colony) characteristics were studied and recorded. Selected bacterial colonies were transferred into slope of the slants prepared with the corresponding plating media for further studies. The culture tubes (slant tubes) were kept in polythene bags. The bags were tied up and preserved as stock culture in a refrigerator at 2 to 8°C. These isolates were transferred to fresh medium periodically. When all plate shown only one type of colony distinctly, it was considered as pure. Uniform vegetative and reproductive structures were also indicative of purification of the isolates. For bacteria, isolates were purified by repeated streaking on to nutrient agar plate. The pure culture of the isolates was coded according to the number of colonies and the serial of the sample used. The code numbers were maintained and followed till identification of the isolates after through characterization.

Morphological characters of the selected isolates can be observed by culture and microscopic methods. By the culture method, colony characteristics on agar

plate, agar slants, and growth in liquid or in deep media were observed. But microscopic methods such as: Gram's staining and acid fast-staining generally carried out for demonstration of size, shape, arrangement and color of isolates. Finally, 6 strains of bacteria were selected by comparing their growth, color and size of the colony in culture media and on the basis of their morphology and nature of arrangement through microscopic study.

Biochemical identification of the selected strains

Identification of bacteria was performed on the basis of cultural characteristics and colony morphology on the Nutrient agar, agar slant and TCBS agar. Gram's staining, acid fast staining (Zeihl-Neelson, 1883), motility test (Eklund and Lankford, 1967), sugar fermentation test (SAB, 1957) and biochemical tests such as: oxidase test (Collins and Lyne, 1984), catalase test (Claus, 1995), gelatin hydrolysis test (Collins and Lyne, 1984), citrate utilization test (Collins and Lyne, 1984), indole test (SAB, 1957), Voges-Proskaur (VP) test (Bryan, 1950),methyl red reaction (Bryan, 1950) and production of hydrogen sulphide (Bryan, 1950) were performed to identify bacteria.

Results

In this study, the total bacterial count of the collected samples range was 26 $\times 10^3$ to 84 $\times$ 10^3cfu/ml. The total bacterial count was determined by serial dilution and pour plate method on nutrient agar plate. The total bacterial counts of the collected samples are shown in Table 1.

Table 1. Total bacterial count of the samples.

Sample No.	Total bacterial count (cfu/ml) (x 10^3)
1.	81
2.	69
3.	84
4.	26
5.	39
6.	75

During the period of isolation two types of media were used, e.g. nutrient agar and TCBS agar for isolating bacteria. Primarily, a total number of 6 bacterial colonies were isolated on the basis of their colony morphology. Out of the 6 isolates, 2 bacterial isolates were selected for further study on the basis of their staining properties, cell form and cell arrangement. Finally, 2 bacterial isolates were selected for further study on the basis of their physiological and cultural characteristics. In this study, 2 bacterial isolates were found to produce comma shaped form with single arrangement and in staining both bacterial isolates were found to be Gram negative and non- acid fast (Table 2).

Table 2. Microscopic feature and staining character of the selected isolates of bacteria.

No. of Isolates	Vegetative cell		Staining	
	Form	Arrangement	Gram	Acid fast
SIVI	Comma shaped	Single	Negative	Non-acid fast
S1V2	Comma shaped	Single	Negative	Non- acid fast

In the present study, one shrimp isolate was found to produce yellowish color colony on TCBS agar plate which is designed as S1V1 and other shrimp isolate was found to produce green color colony on TCBS agar plate which is designed as S2V2 (Fig. 1).In this study, diffuse growths on MIU medium were found indicating that the organisms were motile (Fig. 2). Shrimp isolate which is designed as S1V1 fermented mannitol and sucrose and produced acid but shrimp isolate which is designed as S2V2 did not ferment sucrose in present research work (Table 3 and 4).

To identify the bacteria at species level several biochemical tests were carried out. In the present research work, shrimp isolates were found positive for indole, methyl-red, citrate, catalase and oxidase tests and were found negative for voges-proskauer, gelatine hydrolysis and H_2S production tests (Table 3 and 4).

Table 3. Morphological, cultural, physiological and biochemical characteristics of the bacterial isolate SIVI collected from Chapira, Assasuni, Satkhira.

Parameters	Observations	References	Interpretation
Vegetative cell	Comma shaped	Bergey's Manual of Determinative Bacteriology, 8th ed. (Buchanan and Gibbons, 1974)	*Vibrio nereis*
Cell arrangement	Occur singly or in pair		
Gram staining	Gram negative		
Acid fast staining	Non acid fast		
Nutrient agar colonies	Form: spherical, Margin-entire, Elevation: raised, Surface: smooth		
Colony color on TCBS agar	yellow to green		
Catalase test	Positive		
Motility test	Positive		
Gelatin hydrolysis	Negative		
Citrate utilization test	Positive		
Voges-Proskauer (VP) test	Negative		
Methyl red (MR) test	Positive		
H_2S production	Negative		
Indole test	Positive		
Oxidase test	Positive		
Fermentation test	Mannitol: Acid without gas Sucrose: Acid without gas		

Growth response at different Temperature (°C)

10°C	27°C	37°C	45°C
-	++	+	--

Legend: ++= Strongly positive, += Moderate, --= Strongly Negative, - = Negative.

Table 4. Morphological, cultural, physiological and biochemical characteristics of the bacterial isolate S1V2 collected from Bausuli, Assasuni, Satkhira

Parameters	Observations	References	Interpretation
Vegetative cells	Comma shaped	Bergey's Manual of Determinative Bacteriology, 8th ed. (Buchanan and Gibbons, 1974)	*Vibrio harveyi*
Cell arrangement	Occur singly		
Gram staining	Gram negative		
Acid fast staining	Non acid fast		
Nutrient agar colonies	Form- spherical Margin-entire Elevation-raised Surface- smooth Color- Yellow		
TCBS	Greenish		
Catalase test	Positive		
Motility test	Positive		
Gelatin hydrolysis	Positive		
Citrate utilization	Positive		
Voges-Proskauer (VP) test	Negative		
Methyl red (MR) test	Positive		
H_2S production	Negative		
Indole test	Positive		
Oxidase test	Positive		
Fermentation test	Mannitol:		

	Acid without gas Sucrose: No fermentation		
Growth response at different Temperature (°C)			
10°C	27°C	37°C	45°C
-	++	-	--

Legend: ++= Strongly positive, -- = Strongly Negative, - = Negative.

Biochemical and sugar fermentation results of shrimp isolates identify the shrimp isolate S1V1 as *Vibrio nereis* and V2S2 shrimp isolate as *Vibrio harveyi* (Table 3 and 4). IMViC tests results of *Vibrio nereis and Vibrio harveyi* are shown in Fig. 2.

Discussion

Vibrio species are widely distributed in culture facilitates throughout the world. Vibrio-related infections frequently occur in hatcheries, but epizootics also commonly occur in ghers reared shrimp species. Vibriosis is caused by gram-negative bacteria in the family Vibrionaceae. In present research work, *V. nereis* and *V. harveyi* were identify in *Penaeus monodon* shrimp culture in shrimp ghers. Vibriosis is caused by a number of Vibrio species of bacteria, including: *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida* which reported by Brock and Lightner, 1990; Ishimaru *et al.*, 1995. *V. anguillarum*, *V. campbelli*, *V. nereis*, *V. cholerae* non 01 (sucrose-negative) and *V. splendidus* has also been reported in association with disease outbreaks in shrimps (Lavilla-Pitoga, 1990; Sahul-Ha-meed *et al.*, 1996).

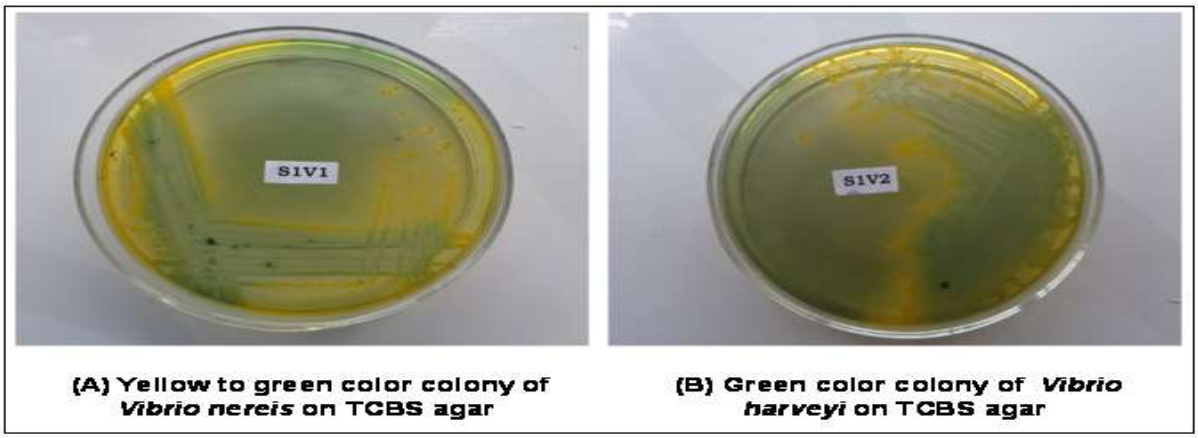

Fig. 1. Cultural characteristic of *Vibrio nereis* (A) and *Vibrio harveyi (*B) on TCBS agar.

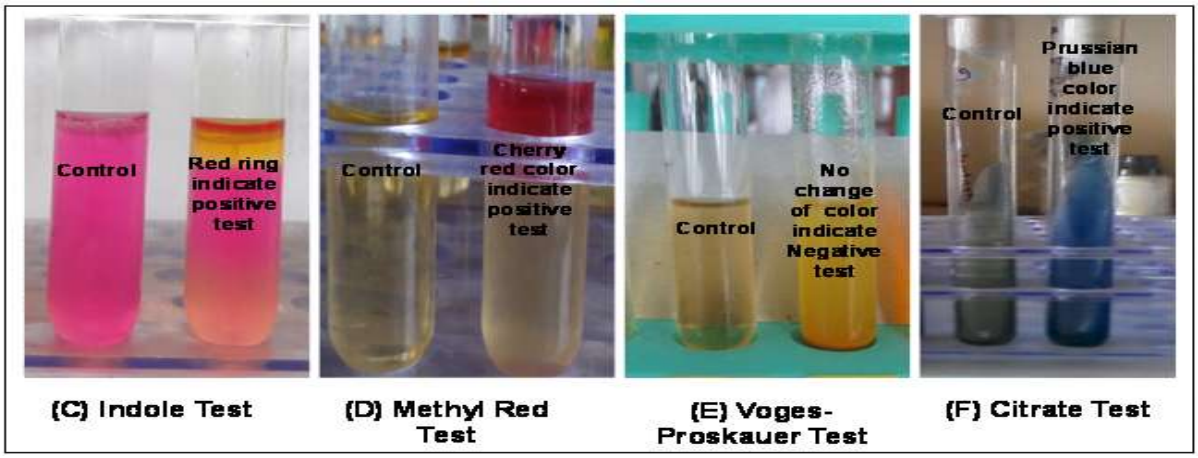

Fig. 2. IMViC test results of *Vibrio nereis* and *Vibrio harveyi*.

In the present study, two bacterial species were isolated from *Penaeus monodon* shrimp belonging to the genera *Vibrio*. The isolates S1V1 and S2V2 were provisionally identified as *V. nereis* and *V. harveyi* respectively on the basis of their cultural, morphological and biochemical characteristics. Similar findings were reported by Buchanan and Gibbons (1974); Rahman *et al.,* (2010). We found *V. nereis* to produce yellow color colony and *V. harveyi* to produce green color colony on TCBS agar plate which was also reported by Buchanan and Gibbons (1974); Rahman *et al.,* (2010).

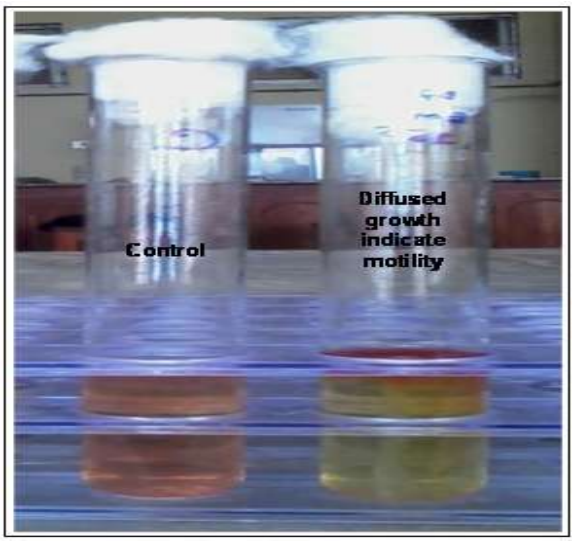

Fig. 3. Motility tests results of *Vibrio nereis* and *Vibrio harveyi.*

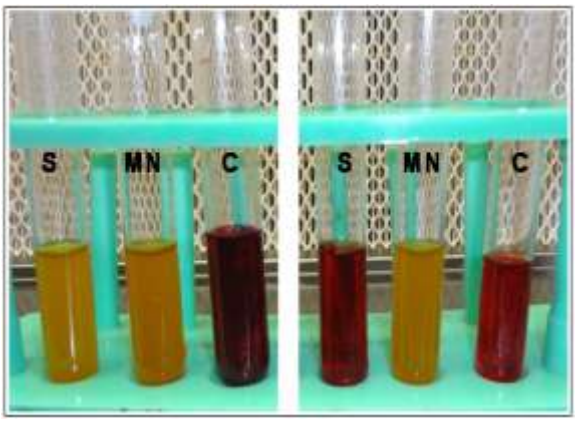

Fig. 4. Results of sugar fermentation tests. (i) *V. nereis* fermented Sucrose (S) and Mannitol (MN) with acid production and no change of color of sugar broth in control (C). (ii) *V. harveyi* fermented Mannitol (MN) with acid production but did not fermented Sucrose (S) and no fermentation is seen in control (C).

Further, on staining, the isolated bacteria appeared Gram negative and comma shape, non-acid fast which was also reported by Buchanan and Gibbons (1974); Rahman *et al.,* (2010). With regard to motility test, both isolates were found motile in MIU medium (Fig. 3). Similar findings were also described by Buchanan and Gibbons (1974); Rahman *et al.,* (2010). In present research work, *V. nereis* was found to ferment sucrose and mannitol and produced acid but not gas. On the other hand, *V. harveyi* found to ferment mannitol with the production of acid only but did not ferment sucrose (Fig. 4).

Both findings are in agreement with Buchanan and Gibbons (1974); Rahman *et al.,* (2010). In this study, *V. nereis* and *V. harveyi* revealed negative reaction in H_2S production and VP tests but in gelatin hydrolysis test *V. nereis* gave negative reaction and *V. harveyi* gave positive reaction. The result of biochemical tests for *V. nereis* and *V. harveyi* revealed positive reaction in oxidase, catalase, citrate, indole and MR tests and all the biochemical tests result in agreement with the findings of Buchanan and Gibbons (1974); Jayasinghe *et al.,* (2008); Rahman *et al.,* (2010).

Conclusion

This study was performed to isolate and identify *Vibrio nereis* and *Vibrio harveyi* from shrimp intestine that are collected from gher of coastal water of Satkhira district of Bangladesh. The present research work indicated that intestine of *Penaeus monodon* shrimp harbor *Vibrio harveyi* and *Vibrio nereis* which might cause Vibriosis in shrimp and public health problem if enter into human food chain.

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