Characterization of Pathogenic Vibrio parahaemolyticus Isolated From Fish Aquaculture of the Southwest Coastal Area of Bangladesh

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Vibrio parahaemolyticus is a major foodborne pathogen responsible for significant economic losses in aquaculture and a threat to human health. Here, we explored the incidence, virulence potential, and diversity of V. parahaemolyticus isolates from aquaculture farms in Bangladesh. We examined a total of 216 water, sediment, Oreochromis niloticus (tilapia), Labeo rohita (rui), and Penaeus monodon (shrimp) samples from the aquaculture system where 60.2% (130/216) samples were positive for V. parahaemolyticus. Furthermore, we identified 323 V. parahaemolyticus strains from contaminated samples, 17 of which were found positive for trh, a virulence gene.

Four isolates out of the 17 obtained were able to accumulate fluid in the rabbit ileal loop assay. The correlation between the contamination of V. parahaemolyticus and environmental factors was determined by Pearson correlation. The temperature and salinity were significantly correlated (positive) with the incidence of V. parahaemolyticus.

Most of the pathogenic isolates (94.1%) were found resistant to ampicillin and amoxicillin. O8: KUT was the predominant serotype of the potentially pathogenic isolates. ERIC-PCR reveals genetic variation and relatedness among the pathogenic isolates. Therefore, this region-specific study establishes the incidence of potential infection with V. parahaemolyticus from the consumption of tilapia, rui, and shrimp raised in farms in Satkhira, Bangladesh, and the basis for developing strategies to reduce the risk for diseases and economic burden.

Keywords: Vibrio parahaemolyticus, aquaculture, rabbit ileal loop, histopathology, trh

INTRODUCTION

The Gram-negative, facultative anaerobic bacterium Vibrio parahaemolyticus is usually present in tropical and temperate coastal waters, as well as in shrimp aquaculture (Heredia et al., 2009). It is indigenous to the estuarine ecosystem and is associated with human gastrointestinal disease, wound infections, and septicemia (Joseph et al., 1982; Wong et al., 2000). V. parahaemolyticus has been considered an etiological agent of foodborne-related illness throughout the world from...
the very beginning of its discovery (Yeung and Boor, 2004). Consumption of raw, parboiled, or contaminated seafood, including shrimp, prawn, fish, and shellfish, is the most common reason for human gastrointestinal infections (Yeung and Boor, 2004; Mahmud et al., 2007; Su and Liu, 2007; Sala et al., 2009; Khouadjia et al., 2013; Khimmakthong and Sukkarun, 2017). A growing number of *V. parahaemolyticus* transmissions and outbreaks engendered by strain members of a pandemic clonal complex have been witnessed all through the world from the early 90s (DePaola et al., 2000; Chowdhury et al., 2004; Ansaruzzaman et al., 2005; González-Escalona et al., 2005; Martínez-Urtaza et al., 2005; Quilici et al., 2005; Fuenzalida et al., 2006).

Vibrio-related infections caused by *V. parahaemolyticus*, which eventually turn into an epidemic, have been observed in Asia, Europe, the United States, Peru, and Chile in the past few decades. These outbreaks were linked with rare, gradual seawater temperature rises along the shoreline (Velazquez-Roman et al., 2014). Several studies performed worldwide implicate environmental factors such as turbidity, temperature, water salinity, amounts of organic matter, and suspended chlorophyll, among others, in the distribution of *V. parahaemolyticus* and the abundance of the species (López-Hernández et al., 2015). Although the mechanism of *V. parahaemolyticus* infection in humans is not well known yet, two hemolysins are broadly accepted as pathogenicity indicators, which are the thermostable direct hemolysin (TDH) and the tdh-related hemolysin (TRH) (West et al., 2013; Jones et al., 2014; López-Hernández et al., 2015). *tdh* is a protein that helps in pore formation, which has been implicated in the invasion of bacteria, and *trh* plays a major role in virulence. Many *V. parahaemolyticus* clinical isolates have *trh* and/or *tdh*; however, a comparatively limited quantity of environmental isolates harbor such genes (López-Hernández et al., 2015; Leoni et al., 2016).

There are 71 K antigens and 13 O antigens that have been recognized and considered related to gastroenteritis by the Serological Typing Committee of *V. parahaemolyticus* (Ishibashi et al., 2000). Typically, in the outbreaks, a wide number of serovars (O3:K6, O4:K68, O1:K25, O1:KUT, O1:K26 etc.) are present (Velazquez-Roman et al., 2014). Post-1996, the growing occurrence of gastroenteritis was associated with the pandemic serotype O3:K6 in most of the world (Okuda et al., 1997; Bag et al., 1999). In recent years, the correlation of this serotype O3:K6 with most of the *V. parahaemolyticus* infections in the United States, South Korea, Thailand, Japan, Laos, Taiwan, and India indicates that this species might have had an uncommon food-borne trait and possess the pandemic ability (Bag et al., 1999). Several molecular typing techniques such as random amplified polymorphic DNA (RAPD), ribotyping, pulsed-field gel electrophoresis (PFGE), and multi-locus sequence typing (MLST) (Bag et al., 1999; Marshall et al., 1999; Wong et al., 1999; González-Escalona et al., 2008; Yang et al., 2008) have been valuable for illustrating the genetic diversity at the strain level (Olive and Bean, 1999). For the subtyping of the *V. parahaemolyticus* species containing strongly conserved repetitive intergenic consensus sequences, ERIC-PCR has been shown to be efficient (De Bruijn, 1992; Chen et al., 2012).

Shrimp export is positioned as one of the leading earning sectors in Bangladesh. Over 40,000 tons of *P. monodon* and *M. rosenbergii* shrimps were exported in 2016 and 2017 along with smaller volume of other species. More than 80% of the shrimp production was shipped to the European Union market, mainly Germany, Netherlands, and Belgium, in 2017. According to the estimation of the fish industry, 5.02 million metric tons of fish production will be achieved within 2020–21. However, the number of detention and rejection cases due to the detection of *V. parahaemolyticus* from Asia is increasing (Sujeewa et al., 2009).

In multiple ecological studies, the relationship of temperature and salinity with *V. parahaemolyticus* has been reported, but the extension of this relationship depends on the region and season (Takemura et al., 2014). Similarly, nutrients, pH, and turbidity were incoherent and depended on the region and the variability of these factors. Therefore, it is necessary to conduct region- and area-specific studies to provide a detailed description of the effect of environmental parameters on *V. parahaemolyticus* concentration (Froelich and Noble, 2016). Consumption of undercooked and raw seafood is the primary way of *V. parahaemolyticus* exposure and infection. Fishes and shrimps can accumulate the bacterium from the aquaculture prior to their harvesting process. As the incidence of *Vibrio* spp. in the environment is unavoidable, their increasing presence in coastal water and sediment create public health issues for the aquaculture industry and the consumers. Therefore, substantial interest has been grown in understanding the biotic and abiotic factors of *V. parahaemolyticus*. Such data has value for estimating the properties and abundance of the species in aquaculture.

Standing on the abovementioned basic contextual information, we conducted a yearlong field-based study to correlate the abundance of non-pathogenic and pathogenic *V. parahaemolyticus* with seasons and other factors such as temperature, pH, and salinity. In this study, pathogenic strains were further characterized to determine their virulence potential in a rabbit model.

### MATERIALS AND METHODS

#### Sample Types, Sources, and Study Sites

Water, sediment, and three different types of fishes (*Penaeus monodon*—shrimp, *Oreochromis niloticus*—tilapia, and *Labeo rohita*—rui) were collected from three sampling sites located in Satkhira Sadar Upazila of Satkhira district (*Figure 1*), one of the major shrimp-harvesting areas of Bangladesh. The sampling was conducted from the period of May 2017–April 2018, once a month. The sampling sites were (1) low-saline aquaculture: shallow water (22° 38’ 44.9″ N 89° 04’ 47.9″ E) (2) brackish aquaculture: enclosed river water (22°39’28.9″N 89°04’30.6″E), and (3) brackish water: flowing river water (22°39’45.9″N 89°04’33.4″E). Water and sediment were taken from all three sites, whereas Tilapia was collected from sites (1) and (2), and rui fish and shrimp were collected only from site (1) and site (2), respectively.
Sample Collection
All the samples were collected in sterile bottles (Nalgene, United States) of 500 ml and 250 ml and a sterile easy-to-close zipper bag (Fisherbrand™ Whirl-Pak™, United States). Water and sediment samples were taken from three different spots of each site, and three pieces of each category of fishes were picked up in every sampling time to cover the maximum areas of the site according to the American Public Health Association (APHA) (Federation and American Public Health Association, 2005). After collection, samples were shipped to the Laboratory of Environmental Health (LEH) at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), in an insulated sample-carrying box maintaining the temperature ranging from 4 to 10°C. Then, the samples were processed within 12 h of collection according to the APHA guideline.

Measurement of Physicochemical Parameters of Water Samples
Salinity and temperature were measured using a conductivity meter (Sens-Ion 5, HACH, United States), and pH of the water samples was measured with a pH meter (Sens-Ion+ pH1, HACH, United States) in each site following the manufacturer’s instructions.

Sample Processing
Inoculation of water and sediment samples on Thiosulfate Citrate Bile Salt Sucrose (TCBS) (DIFCO, BD, United States) agar was done by the spread plate method. Fish samples were washed with sterile 3% NaCl solution to remove surface microorganisms, debris, sand, and slime. Then, the flesh, gut, and gill of the tilapia and rui samples and the flesh and chitin of the shrimp samples were separated aseptically. After that, 5 g of each separated part was homogenized in 45 ml of 3% NaCl solution separately in autoclaved 4 Oz bottles. Then, fish homogenates, water, and sediment samples were enriched in alkaline peptone water (APW) for 18 h. The enrichment broths were then subcultured onto TCBS agar plates and incubated at 37°C overnight. Later on, 4–6 presumptive green or blue-green colonies of 2–3 mm in diameter from each sample were transferred to fresh TCBS agar plates and Chromogenic Vibrio Agar (CVA) (CHROMagar, Paris, France) plates (Hara-Kudo et al., 2001). Isolates showed green to blue green colonies in TCBS, and violet colonies in CVA plates were selected as *V. parahaemolyticus*. Subsequently, the selected strains were inoculated onto Gelatin Agar (GA) plates to see the gelatinase activity for further confirmation. The isolates were preserved at −80°C in LB broth with 30% glycerol in the icddr,b central bio-repository system and in T1N1 soft agar media at room temperature (Alam et al., 2009). For each experiment, the isolates were subcultured on GA plates from the stocks and kept at 37°C for overnight incubation.

Polymerase Chain Reaction (PCR) Assay
Extraction of chromosomal DNA was done using the boil DNA extraction method according to the procedure reported by Mahmud et al. (2020). Presumptively identified isolates were further confirmed by simplex PCR for the *tlh* gene, a species-specific gene for *V. parahaemolyticus* (Taniguchi et al., 1986; Bej et al., 1999; McCarthy et al., 1999; Jones et al., 2012), and the presence of 12 virulent genes was also examined. The presence of all these genes was tested by performing simplex PCR. PCR assays were carried out to detect *tdh* and *trh* genes described by Bej et al. (1999); the GS, PGS, orf-8, and HU-α genes described by Li et al. (2016); and the *ure*, *Mtmase*, VPA 1339 (escC, vscC2), VPA
1346 (yopP, vopP, vopA/P), VPA1321 (vopC), and VPA 1376 genes reported in Caburlotto et al. (2009). All the primers’ sequence and band size are listed in Table 1. The PCR-amplified products were separated in an agarose gel of 1.5% stained in MIDORI green advance and visualized under UV light with a GelDoc Go imaging system (Bio-Rad, United States).

Species Confirmation by VITEK2
All 17 tlh and trh gene-positive V. parahaemolyticus isolates were further confirmed by the VITEK 2 system (bioMérieux, Marcy l’Etoile, France) using a VITEK 2 GN ID card. V. parahaemolyticus (ATCC BAA-238) was used as a positive control for the identification in this system. For VITEK 2 assays, pure isolates were streaked on GA plates and incubated at 37°C overnight. 1–3 isolated colonies were selected from each GA plate and suspended in saline for preparation of inoculum to obtain an absorbance of ~0.5 McFarland Units before being subjected to VITEK 2 analysis.

Antimicrobial Susceptibility Testing (AST) and Calculation of Multiple Antibiotic Resistance (MAR) Indexes
By following the recommendation of the Clinical and Laboratory Standards Institute (CLSI) guidelines (Patel et al., 2015), the pattern of antibiotic susceptibility for 17 trh-positive isolates was obtained by disk diffusion technique. A bacterial suspension with turbidity comparable to 0.5 McFarland standard was made from bacterial inocula taken from overnight cultured Gelatin Agar (GA) plate and swabbed onto the Mueller-Hinton Agar (MHA) (Difco Detroit, MI, United States). Total 15 antibiotic discs (Oxoid, United States) including amoxicillin (AML; 10 µg), ampicillin (AMP; 10 µg), cefotaxime (CTX; 30 µg), ceftriaxone (CRO; 30 µg), cefixime (CFM; 5 µg), cefoxitin (FOX; 30 µg), ciprofloxacin (CIP; 5 µg), gentamicin (CN; 10 µg), nalidixic acid (NA; 30 µg), streptomycin (S; 10 µg), tetracycline (TE; 30 µg), trimethoprim/sulfamethoxazole (SXT; 25 µg), piperacillin-tazobactam (TZP; 110 µg), imipenem (IPM; 10 µg) were used. The inhibition zone surrounding each disk was measured in millimeter (mm) and documented. As per CLSI guidelines, each bacterial species was categorized as resistant (R), intermediate (I), or susceptible (S) (Clinical and Laboratory Standards Institute [CLSI], 2017). The Escherichia coli ATCC 25922 strain was used as a negative control. Calculation of the MAR index was made using formula \( x/y \), where \( x \) is the antimicrobial number to which an isolate became resistant and \( y \) indicates the antimicrobial number to which the isolate got exposed (Krumperman, 1983).

Serotyping
Serotyping of V. parahaemolyticus toxigenic strains was performed utilizing commercially available V. parahaemolyticus
antisera test kit (Denka Seiken, Tokyo, Japan), as defined by Shinoda et al. (1983). The strains were first grown on 3% NaCl-containing Luria-Bertani agar. After incubating for 24 h at 37°C, one loopful inoculum was taken and mixed with 1 ml of normal saline. After boiling for 2 h according to the manufacturers’ instruction, the cell suspension aliquots containing normal saline were utilized for serotyping on the basis of O antigen. The leftover cell suspension (not boiled) was used for K antigen-dependent serotyping.

**ERIC-PCR and Genetic Fingerprinting Analysis**

To determine the clonal relationship among the isolates based on Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences PCR, two primer sequences ERIC1 (5’-3’ATGTAAGCTTCGGGATTTCAC) and ERIC2 (5’-3’ AAGTAAGTGACTG GGTGAGCG) (Versalovic et al., 1991) were used. The conducted amplification conditions were reported by Rivera et al. (1995). Separation of 20 µl PCR product on a 2% agarose gel was performed at 54 volts for 120 min. 1 kb Plus ladders from Invitrogen (Thermo Fisher Scientific, United States) were used as DNA marker. The gel imaging analysis was performed by BioNumerics software (version 4.5) (Applied Maths, Belgium) using the dice coefficient and unweighted-pair group method where average linkages were used to create dendrogram with 1.0% tolerance values.

**Rabbit Ileal Loop Study**

The rabbit ileal loop assay was performed to examine the induction of fluid accumulation as previously described (Twedt et al., 1980), with minor modifications. The experiment was carried out in duplicate with 1.8–2.2 kg adult New Zealand white rabbits. The pathogenic strains of *V. parahaemolyticus* (10⁸ CFU/ml) were injected into the ligated ileal loops of rabbits, which was followed by measurement of the fluid accumulation in each loop at 18 h after injection. The fluid accumulation (FA) ratio was calculated by dividing fluid accumulation (in ml) in each loop by the length (in cm) of the loop. Phosphate-buffered saline (PBS) was used as a negative control, and *V. cholerae* O1 was used as a positive control in this study. Animal Experimental Ethics Committee of International Centre for Diarrhoeal Disease Research, Bangladesh has approved this rabbit ileal loop study (permit number PR-20114).

**Histopathology**

Histopathological examination of the intestinal tissue of the rabbits was also performed to observe the histopathological features of the intestinal wall. Each ligated intestinal loop was washed in phosphate-buffered saline (PBS), and appropriately 2 cm in length was sectioned and fixed in 10% formalin for histopathological examination under a light microscope. Tissues were dehydrated in a series of graded alcohols, further processed, embedded in paraffin, and mounted into paraffin blocks. Staining of the 4 µm tissue sections was performed with hematoxylin and eosin (H&E). The protocols for H&E staining were as described by Bancroft and Gamble (2008).
Statistical Analysis

Pearson correlation analysis was done to observe the relationship between *V. parahaemolyticus* contamination and physicochemical parameters. On the other hand, logistic regression analysis was performed to see the relationship between *trh*-positive *V. parahaemolyticus* and physicochemical parameters. The odds ratio was calculated from the coefficients of the model to identify significant risk factors. The desired statistical significance level was set at 0.05. The analysis was performed by programming language R (R Core Team, 2013) and statistical software package STATA 13 (StataCorp, 2014).

RESULTS

Variations Among the Sites and Samples

A total of 216 samples from 3 different sites [96 samples from site (1), 96 samples from site (2), and 24 samples from site (3)] were analyzed where 60.2% (130/216) samples were positive for *V. parahaemolyticus*.
V. parahaemolyticus, and 7.69% (10/130) of total contaminated samples contained trh gene. Later on, from 130 contaminated samples, 323 representative V. parahaemolyticus strains were obtained, of which 17 strains were found positive for the trh gene. The prevalence of V. parahaemolyticus in contaminated samples was highest in samples collected from site (2) at 81.25% (78/96), closely followed by site (3) at 79.16% (19/24). Site samples was highest in samples collected from site (2) at 81.25% gene. The prevalence of V. parahaemolyticus obtained, of which 17 strains were found positive for the trh gene. Later on, from 130 contaminated V. parahaemolyticus samples, 323 representative V. parahaemolyticus strains were confirmed as V. parahaemolyticus species by using the trh species-specific primer (Supplementary Figure S1) and the VITEK 2 system. Among the trh-positive isolates, 6 (35%), 6 (35%), 4 (24%), and 1 (6%) were from sediment, rui, tilapia, and

### Molecular and Phenotypic Confirmation of the Isolates

The presence of tlh (species-specific gene) confirmed the isolates of V. parahaemolyticus. The occurrence of 12 virulence and virulence-associated genes was detected by PCR analysis of the isolates. Most of the isolates were negative for the genes tested. However, 5.26% (17/323) isolates were positive for the trh gene, a major virulence gene for V. parahaemolyticus, considered as pathogenic. All the 17 tlh and trh gene-positive isolates were further confirmed as V. parahaemolyticus species by using the tlh species-specific primer (Supplementary Figure S1) and the VITEK 2 system. Among the trh-positive isolates, 6 (35%), 6 (35%), 4 (24%), and 1 (6%) were from sediment, rui, tilapia, and

### TABLE 5 | Antimicrobial resistance profiles of V. parahaemolyticus isolates.

| Antimicrobial agents          | Number (%) of S | Number (%) of I | Number (%) of R |
|-------------------------------|-----------------|-----------------|-----------------|
| Amoxicillin (AML)             | 0(0.0)          | 1(5.9)          | 16(94.1)        |
| Ampicillin (AMP)              | 0(0.0)          | 1(5.9)          | 16(94.1)        |
| Ceftriaxone (CRO)             | 12(70.6)        | 2(11.8)         | 3(17.6)         |
| Cefotaxime (CTX)              | 11(64.7)        | 1(5.9)          | 5(29.4)         |
| Cefixime (CFM)                | 11(64.7)        | 6(35.3)         | 1(5.9)          |
| Cefoxitin (FOX)               | 14(82.4)        | 3(17.6)         | 0(0.0)          |
| Meropenem (MEM)               | 15(88.2)        | 2(11.8)         | 0(0.0)          |
| Imipenem (IPM)                | 17(100.0)       | 0(0.0)          | 0(0.0)          |
| Nalidixic acid (NA)           | 17(100.0)       | 0(0.0)          | 0(0.0)          |
| Ciprofloxacin (CIP)           | 15(88.2)        | 2(11.8)         | 0(0.0)          |
| Gentamicin (CN)               | 14(82.4)        | 1(5.9)          | 2(11.8)         |
| Streptomycin (S)              | 16(94.1)        | 0(0.0)          | 1(5.9)          |
| Sulfamethoxazole-trimethoprim (SXT) | 17(100.0)       | 0(0.0)          | 0(0.0)          |
| Tetracycline (TE)             | 17(100.0)       | 0(0.0)          | 0(0.0)          |
| Piperacillin-tazobactam (TZP) | 17(100.0)       | 0(0.0)          | 0(0.0)          |
TABLE 6 | Antibiograms and multiple antimicrobial resistance (MAR) indices of *V. parahaemolyticus* strains.

| Antibiotic-resistant pattern | Isolates | Total antibiotic resistance | No. of isolates | MAR index |
|-----------------------------|----------|----------------------------|----------------|-----------|
| AML-AMP-CRO-CTX             | R06-107  | 4                          | 1              | 0.27      |
| AML-AMP-CN-CTX              | R06-106  | 4                          | 1              | 0.27      |
| AML-AMP-CTX                 | R06-75, R06-99, R09-14 | 3                         | 3              | 0.20      |
| AML-AMP-CN                  | R06-70   | 3                          | 1              | 0.20      |
| AML-AMP-CRO                 | R06-100, R06-101 | 3                        | 2              | 0.20      |
| AML-AMP-S                   | R11-40   | 3                          | 1              | 0.20      |
| AML-AMP                     | R01-15, R01-54, R01-55, R09-20, R10-16, R12-76 | 2                  | 6              | 0.13      |
| AML                          | R06-88   | 1                          | 1              | 0.07      |
| AMP                          | R06-102  | 1                          | 1              | 0.07      |

TABLE 7 | List of *trh*-positive *V. parahaemolyticus* isolates.

| Serial no. | Strain ID | Source | Site no. | Time of collection | Serology | Resistant profile |
|------------|-----------|--------|----------|--------------------|----------|------------------|
| 1          | R01-15    | Sediment | 3       | May 2017          | O11: KUT | AML-AMP          |
| 2          | R01-54    | Sediment | 2       | May 2017          | O5: KUT  | AML-AMP          |
| 3          | R01-55    | Sediment | 2       | May 2017          | O11: KUT | AML-AMP          |
| 4          | R06-70    | Tilapia (flesh) | 1    | October 2017     | O8: KUT  | AML-AMP-CN       |
| 5          | R06-75    | Tilapia (gill) | 1   | October 2017     | O8: KUT  | AML-AMP-CTX      |
| 6          | R06-88    | Tilapia (gut) | 1   | October 2017     | O3: KUT  | AML              |
| 7          | R06-99    | Rui (gill) | 1     | October 2017     | O8: KUT  | AML-AMP-CTX      |
| 8          | R06-100   | Rui (gill) | 1     | October 2017     | O8: KUT  | AML-AMP-CRO      |
| 9          | R06-101   | Rui (gill) | 1     | October 2017     | O8: KUT  | AML-AMP-CRO      |
| 10         | R06-102   | Rui (gut) | 1      | October 2017     | O8: KUT  | AMP              |
| 11         | R06-106   | Rui (gut) | 1      | October 2017     | O8: KUT  | AML-AMP-CN-CTX   |
| 12         | R06-107   | Rui (gut) | 1      | October 2017     | O8: KUT  | AML-AMP-CRO-CTX  |
| 13         | R09-14    | Sediment | 2      | January 2018     | O1: KUT  | AML-AMP-CTX      |
| 14         | R09-20    | Sediment | 2      | January 2018     | O3: K20  | AML-AMP          |
| 15         | R10-16    | Water    | 3      | February 2018    | O5: KUT  | AML-AMP          |
| 16         | R11-40    | Sediment | 3      | March 2018       | O5: KUT  | AML-AMP-S        |
| 17         | R12-76    | Tilapia (gill) | 2 | April 2018      | O5: KUT  | AML-AMP          |

Antibiogram

Antibiotic susceptibility test (AST) was performed on isolated pathogenic *V. parahaemolyticus* using 15 antibiotics selected from different groups (*Supplementary Table S2*). Referring to Table 5, *V. parahaemolyticus* isolates were the most resistant toward ampicillin and amoxicillin (94.1%), followed by cefotaxime (29.4%), and ceftriaxone (17.6%). Some of the antibiotics, such as gentamicin and streptomycin, had a slight resistance of about 11.8% and 5.9%, respectively. All other examined isolates were sensitive to imipenem, sulfamethoxazole-trimethoprim, nalidixic acid, tetracycline, and piperclillin-tezobactam. The next-highest susceptibility rates were found for streptomycin (94.1%), meropenem (88.2%), ciprofloxacin (88.2%), gentamicin (82.4%), cefoxitin (82.4%), ceftriaxone (70.6%), and cefixime (64.7%).

The MAR index was obtained by considering the relation between the number of antibiotics that an isolate is resistant and the total number of antibiotics used. The MAR index range was from 0.07 to 0.27 (Table 6). The maximum MAR index was obtained from two isolates (R06-106, R06-107) from the gut of rui fish, which showed resistance to four antibiotics.

Serotyping Analysis

All the 17 pathogenic *V. parahaemolyticus* isolates were serotyped and categorized into 5 different O groups (O1, O3, O5, O8, and O11). Groups of O8 (47.1%), O5 (23.5%), O11 (11.76%), O3 (11.76%), and O1 (5.88%) were found in this study. Among the isolates, only one was determined by specific K-typing, whereas the rest of the isolates were K-typing untypable. O8: KUT (with eight isolates) was the most common serotype among them. Other O: K serogroups included four O5: KUT, two O11: KUT, one O3: KUT, O1: KUT, and O3: K20 each (Table 7).

ERIC-PCR

ERIC-PCR of the 17 pathogenic isolates resulted in 11–25 amplification bands with a molecular size ranging from 100 to 3,000 bp, where 420, 520, and 1,500 bp were common to most of the isolates. However, considering the similarity coefficient of 0.68, the isolates were divided into 7 clusters designated as A, B, C, D, E, F, and G. Most of the isolates were distributed between the C...
and D clusters. ERIC-PCR banding patterns showed that all the isolates were genetically diverse except one isolate pair (R06-70 and R06-75). Only one strain (R11-40) from sediment is clustered in group F. The reference strain ATCC BAA-238 was in cluster G alone (Figure 5).

Ileal Loop Study and Histopathology

Four out of 17 pathogenic *V. parahaemolyticus* showed fluid accumulation in the rabbit ileal loop. The fluid accumulation (FA) ratio was 2.0, 1.4, 1.6, and 2.2 ml/cm in strains R01-15, R06-75, R06-101, and R12-76, respectively (Figure 6). Among the four strains, R06-101 showed more serosal hemorrhage than the other three strains, characterized by gross bloody mucous in the accumulated fluid. Histopathological features of all four strains showed nearly similar findings, including a moderately degenerated, damaged, and sloughed ileal mucosa (Figure 7; Inside Square); significant/obvious obstruction of blood vessels and infiltration of polymorphonuclear neutrophils (PMN) in the lamina propria. At higher magnification (Figure 7; Inside Circle).

DISCUSSION

The occurrence of *V. parahaemolyticus* has been reported previously in temperate and tropical coastal waters as well as in aquaculture (Heredia et al., 2009; Pui et al., 2014). The incidence of *V. parahaemolyticus* in shrimp, water, fish, and sediments was investigated all year round using cultural and molecular methods. In our study, *V. parahaemolyticus* was present year-round in site (2) and site (3), whereas it was absent in site (1) during the winter-period. Surprisingly, the presence of the pathogenic (trh) gene was more frequent during October from the rui fish sample than others. The seasonal and geographical distribution of *V. parahaemolyticus* has been considered to be significantly dependent on temperature in temperate zone shellfish-growing areas (Kaneko and Colwell, 1975; Kiiyukia et al., 1989; Mahmud et al., 2006).

Based on the year-long assessment of water sample salinity gradients, for which sample collection was done from the three study locations, a salinity-dependent area demarcation can then be made. During summer, maximum salinity was encountered in site (2) and site (3) due to an increased brackish water inflow, whereas in site (1), low salinity was present throughout the year as it was collected from underground shallow water, which is not affected by seawater. Contrariwise, minimum salinities in site (2) and site (3) were reported in the rainy season due to heavy rainfalls associated with the blending of floodwater from the nearby regions. At all three study locations, the temperature of water varied consistently, the highest temperature was reported in the summer, followed by the rainy season, and the lowest in the winter; the pH was somewhat unchanged (7.99 ± 0.31) within its intrinsic alkaline status. However, in the rainy season, the maximum alkalinity may be due to the floodwater inflow from the adjacent areas containing a significant amount of organic residues.

Statistically, the salt tolerance potential (at a salt concentration of 2–10%) can be due to a crucially significant positive correlation between salinity and *V. parahaemolyticus* (Table 3; Caburlotto et al., 2010; Johnson et al., 2010; Whitaker et al., 2010) but a
significant negative association was found between salinity and the pathogenic V. parahaemolyticus, as most of these pathogenic isolates were found in the samples (tilapia, rui) of the site (1), which was quite a low-saline site.

In multiple previous studies, trh-positive V. parahaemolyticus were more frequent than tdh positive in the environmental samples (Bauer et al., 2006; Ellingsen et al., 2008). Although only 1–2% of environmental V. parahaemolyticus strains harbor tdh and/or trh gene, this small amount is sufficient to have a significant impact on public health in tropical developing countries (Cook et al., 2002). In our study, 5.26% (17/323) of the V. parahaemolyticus strains showed pathogenic potential, by the presence of trh gene, but all the isolates were negative for the tdh gene. This result appears similar to the previous study by Letchumanan et al. (2015) but contrary to the earlier reports where a high prevalence of these virulence factors was observed in V. parahaemolyticus strains in natural estuarine and coastal waters (Johnson et al., 2012; Akther et al., 2016).

In this study, V. parahaemolyticus isolates exhibited a high level of resistance to amoxicillin and ampicillin. Resistance to penicillin could probably be due to the extensive use of antibiotics in aquaculture and the effect of residual antibiotics in aquaculture systems. Therefore, the penicillin group is considered ineffective for the management of V. parahaemolyticus infections. However, most of the isolates were susceptible to the majority of the antibiotics used. Susceptibility patterns of V. parahaemolyticus isolates to different antibiotic groups such as quinolone, tetracycline, carbapenem, aminoglycosides, and cephalosporin were analogous with other studies reported in various sample sources (Lesmana et al., 2001; Han et al., 2007; Yano et al., 2011; Ottaviani et al., 2013). In the current study, the MAR index value was observed greater than 0.2 in 8% (2/17) of pathogenic isolates, which indicates that samples originated from a high-risk contamination source where numerous antibiotics are used (Krumperman, 1983). Aquatic bacteria are subjected to environmental contaminations in coastal and estuarine waters, particularly through wastewater treatment plants and agricultural activities.

**FIGURE 6** | Rabbit ileal loop assay to assess enterotoxicigenic activity. Pictorial view of rabbit ileal loop of different environmental strains. Analysis of fluid accumulation of different V. parahaemolyticus strain isolated from the environment (D). Rabbit ileal loops were inoculated with 10⁸ CFU/ml of each trh-positive V. parahaemolyticus strain (A–C). Results are expressed as fluid accumulation (FA; in milliliters) per loop length (in centimeters). PBS and the V. cholerae O1 were taken as controls in this assay.
runoff, which might carry a different amount of antimicrobials and heavy metals and subsequently give selective pressure to the aquatic bacteria to be antimicrobial-resistant (Stepanauskas et al., 2006; Gordon et al., 2007). Therefore, continuous surveillance and monitoring programs are essential for both the prevalence and the antimicrobial susceptibility profile to ensure aquaculture safety.

The pathogenic strains were serotyped for epidemiological purposes. The majority of those strains contained O8 antigen, followed by O5, O11, O3, and O1. Most of the strains could not be typed serologically for the K antigen using conventional kits. In our study, serotype O8: KUT was the most frequent, which was also found in the environmental samples in several other studies (Pal and Das, 2010; Jones et al., 2012; Zhang et al., 2013; Haley et al., 2014). Other serotypes found in this study are O5: KUT, O11: KUT, O3: KUT, O1: KUT, and O3: K20. Interestingly, serotype O1:KUT, a serovariant of O3:K6 (González-Escalona et al., 2008), played a major role in causing a massive outbreak of cholerae in Kolkata, India, and afterward found in Asia, Africa, Europe, Latin America, and the United States (Okuda et al., 1997).

The isolates of this study were categorized into 7 clusters at a similarity of 68% based on the ERIC-PCR approach. The clustering of 5 pathogenic strains out of 17 suggested their genetic linkage though they were collected from different sources at a different time interval. Unlike the other strains in this experiment, the ATCC BAA-238 reference strain only belonged to cluster G. One sediment-derived isolate (R11-40) was placed into the cluster F alone and was distinct from the rest of the isolates genetically. ERIC-PCR-based clustering did not correlate with sources of isolation, time, and serotyping. This finding is similar to that of other research, demonstrating the genetic variation within the strains of V. parahaemolyticus (Wong et al., 1999, 2000; Chen et al., 2012; Lu et al., 2016).

Approximately 24% of the pathogenic isolates found in the animal study were associated with fluid accumulation, with/without leading to the death of the rabbit, which implies that all the pathogenic isolates were not able to accumulate fluid in the rabbit intestine. A correlation between enterotoxigenicity of V. parahaemolyticus strains and virulence potential was reported in earlier studies (Hiyoshi et al., 2010). Thus, the presence of the trh virulence gene could not be linked to enterotoxigenic potential in the present experiment. Xu et al. (1994) showed that the deletion of trh led to a minor but noticeable accumulation of fluid in the ligated rabbit small intestine. These findings reveal that tdh and trh alone do not explain the cytotoxicity and enterotoxicity of pathogenic V. parahaemolyticus and propose that certain other mechanisms that have not yet been discovered may contribute to the accumulation of fluid, excluding the factors examined in this study and other studies to date (Park et al., 2004).

This study was limited to only three aquaculture sites due to funding constraints. If an extensive study including a large number of farms were taken, it would be possible to know the scenario better. However, our study provides comprehensive research on pathogenic V. parahaemolyticus isolated from different aquacultures by highlighting the occurrence, virulence, antibiotic resistance pattern, serotyping, genetic diversity, and

FIGURE 7 | Ileal tissue sections obtained from rabbits infected with 10⁸ CFU/ml of trh-positive V. parahaemolyticus showed severe histopathological changes in the mucosa represented by infiltration with PMN in the lamina propria (inside circle) and degeneration, damage, and sloughing (inside square).
rabbit ileal loop study. This study suggests the presence of pathogenic *V. parahaemolyticus* in the aquaculture sample. Moreover, their resistance to multiple antibiotics could be a threat to public health which should not be ignored. The presence of different serotypes including few pandemic serotypes might indicate the diversity and outbreak potential of these isolates. The clustering of the isolates shows their genetic diversity. The fluid accumulation in the ileal loop of rabbit suggests the pathogenicity of those isolates. These data might be of help in assessing human health risk due to the consumption of fish in the southwestern coastal area of Bangladesh. Subsequently, it can be helpful for public health organizations as a future reference for policymaking guidelines.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Animal Experimental Ethics Committee, IRB Secretariat, Research Administration, CMS, International Centre for Diarrhoeal Research, Bangladesh.

**AUTHOR CONTRIBUTIONS**

ZM, MM, SI, and SA: conceptualization. AS, MM, and SI: methodology. MD and MA: software. ZM, RI, DM, and ZM, MM, SI, and SA: conceptualization. AS, MM, and SI: formalization analysis and data curation. ZM and SI: resources.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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