Section A-Research paper

VIRULENCE CHARACTERISTICS OF VIBRIO PARAHAEMOLYTICUS ISOLATES FROM DIARRHOEAL PATIENTS

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Abstract:

Vibrio parahaemolyticus (*V.parahemolyticus*) has become one of the emerging enteric pathogens linked to gastroenteritis, diarrhea, and food poisoning worldwide. Subjects who presented with the clinical features and symptoms of acute diarrhea were included. Of 325 diarrheal samples, majority of the subjects had diarrheagenic *Escherichia coli* (n = 138), *V.parahemolyticus* (n = 73) respectively. Thermostable hemolysin and motility was seen in all the isolates of *V. parahaemolyticus*. Their susceptibility pattern revealed that 8.2%, 26%, 100% were resistant to norfloxacin, ciprofloxacin and ampicillin respectively. Isolates were detected both by molecular and conventional methods using polymerase chain reaction (PCR). Confirmation of *V. parahaemolyticus* colonies by specific target gene toxR, tdh, trh from the diarrhoeal patients. PCR results showed 100% toxR, 22% tdh and 12.3% trh gene among the isolates. There were limited number of studies found analysing the virulent genes of *V.parahemolyticus* and its effects on human beings.

Keywords: *Vibrio parahaemolyticus*, enteric pathogen, Diarrhea, toxR, tdh, trh.

1. Introduction:

*Vibrio parahaemolyticu*s (*V. parahaemolyticus*) has become one of the enteric pathogens linked to gastroenteritis, and diarrhea world wide.[1] *V. parahaemolyticus* is a gram negative and swarming motile bacilli. It is known to cause foodborne diseases and in many Asian nations, it naturally exists in coastal waters.[2] Its outbreaks are commonly reported in Europe and the United States. It is a type of microorganism with high genetic variability. [3] To detect genetic variability; pulsed-field gel electrophoresis, multilocus sequence typing, serological typing, and whole-genome sequencing were done. [4].

Serotypes 11 O and 69 K antigens are the most common serotype causing human infection and are used in clinical diagnosis.[5] The rare serovar which spread diarrheal disorders over several nations was O3: K6. A Gram-negative, halophilic bacteria called *V. parahaemolyticus* continues to be one of the principal causes of gastroenteritis and diarrhoea. It is believed that haemolysin (TDH) and TDH-related hemolysis are essential elements of this bacterial infection.[6]

The directed hemolysin (TDH) found in *V.parahaemolyticu*s may produce two nm-sized gaps in cell membranes, which allows water and ions to freely enter, causing red blood cells to function hemolytically.[7] *V. parahaemolyticus* has been found as the second widespread bacterial pathogen, after diarrheagenic *Escherichia coli* (*E.coli*).[8,9] Thus, in this study, virulence factors were identified and genomic confirmation of *V.parahaemolyticus* from patients with diarrhea were analyzed.

2. Materials and Methods:

In this study, all cases of severe diarrhea initially presented to the outpatient department from January 2021 to December 2021 were included. Stool samples (n=325) were collected and sent to Microbiology Laboratory. All stool samples were subjected to direct microscopy using a wet, saline, and iodine mount to examine the presence of trophozoites or cysts of various diarrheal parasite species. Formol ether sedimentation was carried out and re-examined by microscopy in stool samples with no or low counts of cysts.[10]

The bacterial pathogens were identified by using conventional culture techniques, biochemical and genetic confirmation was done. Samples were cultured on Selenite F Broth, Alkaline peptone broth, MacConkey agar, Salmonella-shigella agar, tryptic soy agar, thiosulphate-citrate bile salts agar at 37 ℃ for 24 hours. (**Figure 1**) Screening of *V. parahaemolyticus* for TDH production was done using Wagatsuma blood agar (Hi-Media).[11]

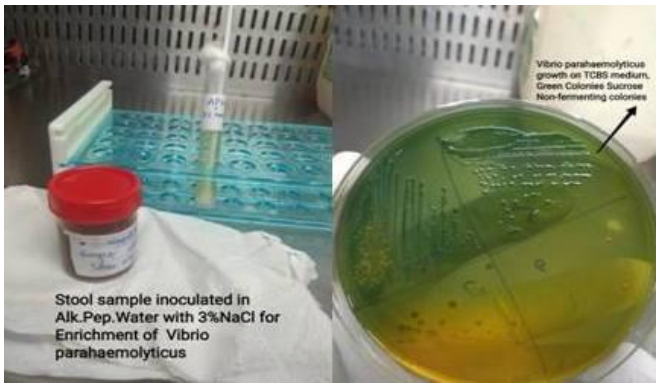

Figure 1: Confirmation of *V.parahaemolyticus*

3. Detection of *V. parahaemolyticus* motility

Grams staining and swarming motility were carried out by adding Bacto agar (6 g) with Luria Bertani (LB) medium (1,000 ml) (Himedia). After 18 hours of culture with LB broth at 37°C of *V. parahaemolyticus* isolates, 0.5 McFarland units concentration was obtained. Swarm zone diameter was measured after adding 2µl of bacterial culture onto the swarming incubation 37°C - 4 and 8 hours.[12,13]

3.1 Biochemical identification

All the isolates of *V. parahaemolyticus* were subjected to Catalase, Cytochrome Oxidase and other biochemical tests. [11,14] **Table 1**. Gram character

3.2 Kanagawa phenomenon

Wagatsuma blood agar (Hi-Media) kept at 37°C, 24 hours to detect the ability to hemolyze erythrocytes and the zone of hemolysis was visually examined. TDH production is indicated by the clear zone of hemolysis surrounding the colony.[11,12]

3.3 Antimicrobial susceptibility test

This test was performed (CLSI, 2017) by disc diffusion method using Mueller-Hilton agar plates[15]. Ciprofloxacin (5 µg), Ampicillin (10 µg), Norfloxacin (10 µg), Chloramphenicol (30 µg), Tetracyclin (30 µg) and Cotrimoxazole (25 µg) were included in the antimicrobial discs.

3. 4 Determination of species-specific and virulent gene

By using PCR, detection of a species-specific gene (toxR) was carried out. [16] Followed by, identification of virulent genes (tdh, trh) were done among toxR gene positive strains.[17,18] The primers used for the identification of these genes were given in **Table 2**.

Table 1: Biochemical identification of *V. parahaemolyticus*

S. No.	Biochemical Reactions	Abbreviation	*V. parahaemolyticus*
1	Catalase test	CAT	(+)
2	Oxidase test	OXY	(+)
3	Urease production	URE	(-)
4	Nitrate Reductuion	NIT	(+)
5	Indole production	IND	(+)
6	Methyl Red reaction	MR	(+)
7	Vogues–Proskawer reaction	VP	(-)
8	Citrate utilization	CIT	(-)
9	Triple Sugar Iron reaction	TSI	(Alk/Acid^{+}, H_2S^{-})
10	H_2S production	H_2S	(-)
11	Arginine dihydrolase	ADH	(-)
12	Ornithine decarboxylase	ODC	(+)
13	Lysine decarboxylase	LDC	(+)
14	β-galactosidase	ONPG	(-)
15	Glucose production	GLU	(+)
16	Mannitol	MAN	(+)
17	Sorbitol	SOR	(-)

4. Molecular identification of virulent genes

DNA extraction from the samples were done by using Qiagen mini Kit. By measuring absorbance wavelengths at 260 nm and 280 nm, the concentration and purity of DNA were assessed.[19,20] Followed by, the integrity of genomic DNA was checked by Gel electrophoresis.[21] Gene Amp PCR 2700 thermocycler (Applied Bio-systems) system was used to conduct the PCR tests. 25 ul PCR reaction mixture of trh, toxR, and tdh contains 2 µl of template DNA and this was subjected to thermocycler with the conditions listed in **Table 2**. By using electrophoresis, PCR products were confirmed by 1.5% agarose gel, visualised with ethidium bromide, and read with a Gel Documentation Imaging System. [21]

Table 2: Primers used for toxR, tdh, and trh [12]

Target gene	**Encoded protein**	**Oligonucleotide**	**Amplification conditions**	**Amplicon size (bp)**
toxRgene	ToxR regulatory protein	F 5'-GTCTTCTGACGCAATCGTTG-3' R 5'-ATACGAGTGGTTGCTGTCATG-3'	95°C-30 sec 63°C-30 sec 72°C-30 sec	368
tdh	Thermostable direct hemolysin	F 5'-CCACTACCACTCTCATATGC-3' R 5'-GGTACTAAATGGCTGACATC-3'	94°C-60 sec 58°C-60 sec 72°C-60 sec	255
trh	TDH-related hemolysin	F 5'-GGCTCAAAATGGTTAAGCG-3' R 5'-CATTTCCGCTCTCATATGC-3'	95°C-60 sec 55°C-60 sec 72°C-60 sec	250

5. RESULTS:

A total of 325 diarrheal samples were received between January 2021 to December 2021 including 207 male and 118 female in the study. The majority of the patients were diagnosed as having Diarrhoeagenic *Escherichia coli* (n=138) *V. parahaemolyticus* (n=73) followed by *Shigella species* (n=12), *Salmonella species* (n=10) and Commensal flora (n=80). **Table 3** lists the different types of microorganisms isolated from stool specimens.The patient who came to the

OPD with gasterentrities depending on the clinical symptoms were admitted for further evaluation and treatment. The patient with stool samples positive for *V. parahaemolyticus* had severe dehydration and abdominal pain.

Table 3: Isolation of Bacterial pathogens from the diarrhoeal sample

Bacterial Pathogens	No of Isolates (%)
Diarrhoeagenic *Escherichia coli*	138 (34.4%)
Vibrio parahaemolyticus	73 (22.5%)
Shigella species	12 (3.6%)
Salmonella species	10 (3%)
Campylobacter species	8 (2.4%)
Aeromonas	5 (1.5%)
Entamoeba histolytica	2 (0.6%)
Giardia lamblia	2 (0.6%)

5.1 Clinical Symptoms of diarrhoeagenic *V.parahemolyticus* and *E.coli*

Among the clinical symptoms, the majority of subjects were presented with diarrhea (n=73), severe dehydration (n = 41) followed by fever (n = 36), abdominal pain (n = 32), vomiting (n = 27), abdominal distension (n = 19), abnormal mental status (n = 17), nausea (n = 15) and drowsiness (n = 2). All the above patients had *V.parahemolyticus* from the stool sample. In diarrhea caused by Diarrhoeagenic *Escherichia coli*, the majority of the subjects had diarrhea (n=138), vomiting (n = 33) followed by abdominal distention (n = 47), severe dehydration (n = 39), abdominal pain (n = 38), nausea (n = 36), drowsiness (n = 24) and fever (n = 23).

5.2 Antibiogram profiles

V. parahaemolyticus isolates showed resistance to ampicillin (n=73), followed by ciprofloxacin (n=19), norfloxacin (n=6), and cotrimoxazole (n=2). The susceptibility pattern of the profile were shown in **Table 4**.

Table 4: List of Antibiogram profile

Antimicrobial agent	*V.parahaemolyticus* (n=73)		
	R- n (%)	I- n (%)	S-n (%)
Ampicillin	73(100)	-	-
Chloramphenicol	-	19 (26)	54 (74)
Ciprofloxacin	19 (26)	11 (15)	43 (59)
Cotrimoxazole	2 (3)	6 (8.2)	65 (89)
Norfloxacin	6 (8.2)	9 (12.3)	52 (71.2)
Tetracycline	-	-	73 (100)

Note: Resistant (R), Intermediate (I), Sensitive (S)

5.3 Virulence-related characteristics:

5.3.1 Detection of TDH

Screening of V. parahaemolyticus isolates was done by the Kanagawa phenomenon for the detection of TDH production. The hemolytic activity (β-hemolysis) against RBCs was evaluated and all *V. parahaemolyticus* isolates (n = 73) were found to be positive.

5.3.2 Distribution of virulence-associated genes

The toxR gene amplification test revealed that all of the isolates which was tested by the PCR were positive for tdh, toxR, and trh genes. All the isolates of *V.parahemolyticus* were positive for tox R gene in which 40% had both tdh(+) and trh(+), 22% had tdh(+) and trh(-), 12.3% had trh(+) and tdh(-), 26% had tdh(-) and trh(-). (**Figure 2**)

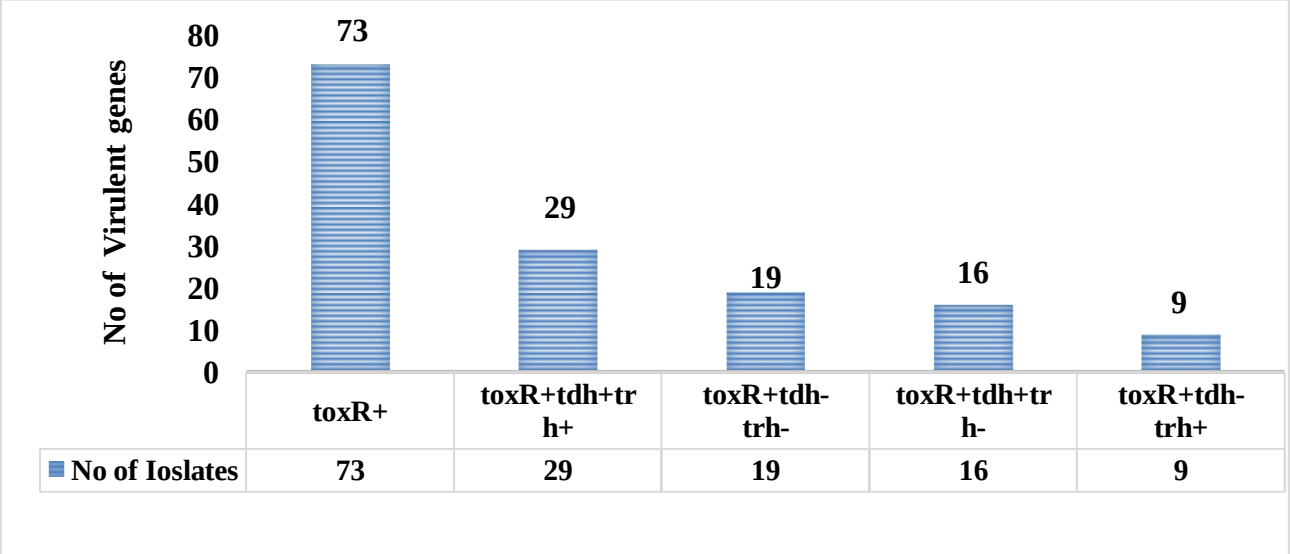

Figure 2: Virulent genes of *V. parahaemolyticus*

6. Discussion:

Diarrhea continues to be one of the largest health issues on the globe today. *V. parahaemolyticus* is an emerging pathogen that causes infectious diarrhea. Following Diarrhoeagenic *Escherichia coli* and *Salmonella* have been exceeded as the most common bacterial cause of food poisoning by *V. parahaemolyticus*. Cases of *V. parahaemolyticus* are commonly recorded throughout the world, but it is found especially in Southeast Asia.[22] All the isolates showed resistance to ampicillin which was found to be similar with the studies documented by Chen *et al.*, [2], and Li *et al.*, [23] whereas Letchumanan *et al.*, documented significant resistance to a range of antibiotics, with 88%, 81%, 70.5%, 73%,51.5% ampicillin, amikacin, kanyamycin, cefotaxime and ceftazidime respectively. [24,25]

The major virulent elements of *V. parahaemolyticus* include hemolysin, direct (tdh) and indirect (trh) heat-resistant hemolysin. In this study, 22% tdh + and trh- were documented which was found to be similar with the study done by Chen *et al.*, [3]

A total of 73 isolates were found to be positive for tox R gene, 22% had tdh(+) and trh(-), 12.3% had trh(+) and tdh(-) which was found to be similar with the study done by Yang *et al* [26], whereas these positivity rates were found to lower with the study done by Sun *et al* [13], in which 84.21% of the isolates were tdh(+) and trh(-), 95.80% were tdh(-), trh(+) revealing

significant pathogenicity. There were limited number of studies found analysing the virulent genes of *V.parahemolyticus* and its effects on human beings. As these strains were responsible for causing life-threatening diarrhea; screening and surveillance of *V.parahaemolyticus* virulence genes has to be improved.

7. Conclusion:

The strains of *V. parahaemolyticus* associated with the genes for tdh and trh causing infection in humans are emerging. It is unclear about the causes for the higher frequency of tdh and trh in clinical strains of *V. parahaemolyticus*. This study help us comprehend the virulence traits and underlying mechanisms of this harmful bacterium. It is necessary to investigate the pathogenic potential of these clinical *V.parahaemolyticus* isolates containing tdh, trh gene. Future research should concentrate on the function of tdh and trh genes in clinical strains of *V. parahaemolyticus* as well as in the environmental strains as these genes were detected more frequently in environmental strains also.

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