

ANTIBIOGRAM AND MOLECULAR CHARACTERIZATION OF *VIBRIO CHOLERAE* ISOLATED FROM MARINE FISH

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Received: 16 February 2017, Revised and Accepted: 11 March 2017

ABSTRACT

Objective: Molecular identification and antibiotic susceptibility evaluation of *Vibrio cholerae* from marine fish available in local fish market Thanjavur, Tamil Nadu, India.

Methods: Inoculation was done using nutrient agar as general media and thiosulfate-citrate-bile salts-sucrose agar as selective media and confirmed as *V. cholerae* by Gram-stain (microscopic observation), growth characteristics of different media, biochemical tests such as methyl red test, nitrate reduction tests, and indole test. Sensitivity (drug sensitivity) was done in Mueller-Hinton agar using disc diffusion method 10 different antibiotics were used to evaluate the antibiogram profile, molecular detection was done by targeting 16S rRNA gene using a universal primer.

Results: *V. cholerae* is present in marine fish samples, as showed by culture method and microscopic observation as well biochemical tests. Polymerase chain reaction (PCR) amplification of 16S rRNA gene showed the amplification of targeted gene and antibiogram profile showed that isolates are more sensitive to ampicillin in comparison with others antibiotics used in this study. Ampicillin can be used for *V. cholerae* infection by the physicians and amoxicillin must be avoided which is resistant.

Conclusion: Molecular detection is safe and rapid methods for bacteria identification as revealed by PCR amplification of 16S rRNA gene. As the isolates are more sensitive to ampicillin in comparison with others antibiotics used in this study. Ampicillin can be used for *V. cholerae* infection by the physicians and amoxicillin, and nitrofurantoin must be avoided.

Keywords: Bacteria, *Vibrio cholerae*, Molecular identification, Universal primer, 16S rRNA gene.

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INTRODUCTION

Cholera continues to be a serious public health problem in developing countries [1] where the disease is endemic, and several outbreaks have been reported. Historical records suggest that cholera-like disease may have been first reported in the Era of Hippocrates (460-377 BC). The geographical distribution of cholera is changing and so is often considered as a re-emerging disease, in part because infections are appearing in novel communities or in communities where the disease had been absent for many years. This may be due to the changes in the environment or climate, following the EL Nino phenomenon which has made conditions favorable for cholera worldwide [2]. The problem of global warming and the inland incursion of sea water covering more and more of the coastal stretches of land could lead us to the brink of a resurgent pandemic. Worldwide there has been increasing in the number of cholera cases and outbreaks in the new communities and with changing profile [3]. During the 19th century, cholera spread across the world from its original reservoir in the Ganges delta in India [4]. The seventh pandemic started in South Asia in 1961 and reached Africa in 1971 and the Americas in 1991. *Vibrio cholerae* O1 El Tor biotype was the causative agent of the seventh pandemic. In 1992, *V. cholerae* O139 was isolated in India and then in Bangladesh. It was the first non-O1 serotype responsible for cholera epidemics. El Tor *V. cholerae* has replaced their classic counterpart over the past few decades [3]. Cholera outbreaks occur seasonally and are associated with monsoon season, warm temperature, heavy rainfall, and increased plankton population. *V. cholerae* has a unique ability to exist in an autochthonous state in the river and brackish water estuaries and coastal waters and can exist in dwarfish forms in response to nutrient deprivation as a viable but non-culturable form. It can exist in the gut and attached to the surface of both freshwater and marine copepods. In addition, the genetic assortments and re-assortments that are going

on in these isolates, equip them appropriately to survive better in the changing environmental conditions [5,6]. In the year 2014, a total of 190-549 cases of cholera were reported from 42 countries with a case fatality rate of 1.17%. This study was conducted with the aim of characterizing molecular and evaluating the antibiotic profile of *V. cholerae* isolated from marine fish.

METHODS

Sample collection and isolation

Marine fish samples were collected from the local fish market in Thanjavur, Tamil Nadu. Four different species marine fishes were collected including *Sardinella longiceps*, *Rastrelliger kanagurta*, *Epinephelus tauvina*, and *Scomberomorus guttatus*. To examine the bacterial organisms in the fishes taken for study, the methods of culture and plating as described by Venkataraman and Sreenivasan (1952) were followed. The tissues of *S. longiceps*, *R. kanagurta*, *E. tauvina*, and *S. guttatus* were dissected and processed. The 25 g portions were cut, homogenized with 225 ml peptone saline water and after serial dilution, they were subjected to the separate culture medium.

Media

Culture technique procedures recommended by Bergey (1948) were followed. The media employed for the isolation of *V. cholerae* were nutrient agar, nutrient broth, Mac Conkey agar, and thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Hi-Media, India) and these were time tested ones in bacterial cultures.

Biochemical characterization

The isolated pathogens were subjected to biochemical tests for further confirmation apart from the culturing methods. The following are the

tests performed during this study oxidase, catalase, indole, methyl red, Vp citrate, triple sugar iron (TSI), urease, and nitrate reduction were carried out following the standard process in the biochemical analysis.

DNA isolation

DNA from the bacterial genome was extracted as per standard proteinase-K digestion method. Bacterial cultures were prepared and suspended in Luria-Bertani broth (Hi-Media, India) and incubated at 37°C, 110 rpm for 12 hrs. The 12-hr old bacterial cells were pelleted at 15,000 × g or 10 minutes and then suspended in Tris-HCl (pH 7.2), 10 mM ethylenediaminetetraacetic acid (EDTA), 250 mM NaCl buffer having 1% sodium dodecyl sulfate (Hi-Media, India). Proteinase-K (Hi-Media, India) was then added to a final concentration of 100 µg/ml and mixed gently. The suspension was incubated at 37°C for 60 minutes. DNA obtained by sequential phenol-chloroform and chloroform-isoamyl alcohol extractions was precipitated by adding 2.5 volumes of absolute ethanol, and DNA was suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA-pH 7.5). DNA was checked for purity by agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification of 16S rRNA gene

PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The universal primers (Forward primer 5'-AGAGTTTGATCTMTGGCTCAG-3' and reverse primer 5'-GGTTACCTTGTACGACTT-3') were used for the amplification of the 16S rRNA gene fragment. The reaction mixture of 50 µl consisted of 10 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µl of ×10 PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl pH-8.3), 200 µM deoxynucleotide triphosphate, 10 p moles each of the two universal primers and 1.5 mM MgCl₂. Amplification was done by initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, the annealing temperature of primers was 55°C for 30 second and extension at 72°C for 1 minute.

The final extension was conducted at 72°C for 10 minutes. Agarose gel electrophoresis of PCR product was carried out as follow 10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under gel documentation system.

Antibiotic sensitivity tests

Antibiotic sensitivity tests were carried on Mueller-Hinton agar (MHA) (Hi-Media, India) plates by Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966) [7] using antibiotic discs (HiMedia, Mumbai, India) penicillin G (10 units), streptomycin (10 µg), chloramphenicol (30 µg), ampicillin (10 µg), amoxicillin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), nitrofurantoin (10 µg), and norfloxacin (10 µg) (Hi-Media, India). Characterization of the strains as susceptible, intermediately resistant, or resistant was based on the size of inhibition zones around each disc as per the manufacturer's instructions, which matched the interpretive criteria as per CLSI guidelines (CLSI, 2007).

RESULTS

In this study, four different fish species were used (*S. longiceps*, *R. kanagurta*, *E. tauvina*, and *S. guttatus*) for the isolation of *V. cholerae* bacteria, where culturing as well biochemical tests were performed for the identification and characterization of isolates, molecular study through partial 16S ribosomal RNA gene amplification was done after DNA isolation and quality checking.

All four marine fishes selected showed the presence of *V. cholerae*. This show that the incidence of *V. cholerae* bacteria isolated is significant in marine fishes. The selective media TCBS agar showed the presence of the *V. cholerae* bacteria yellow colonies is observed on TCBS agar plates after 24 hrs of incubation. The isolates were presumptively identified by biochemical profiling the different reactions and different changes in the media due to the behavior of isolated bacteria

to the subjected media and chemicals present in media different changes were observed and compared to the standards records all the four isolates were positive for tests performed except Gram staining which characterized the isolates as Gram-negative (Table 1), the amplification of 16s rRNA gene is another factor which confirms that the isolates are bacteria there is confirmation of *V. cholerae* (Table 2). Genomic DNA was isolated and characterized by standard proteinase-K digestion method, and its quality was checked using agarose gel methods, PCR was performed using universal primers 27F and 1492R the products were observed as 1500 bp (Fig. 1). Antibiotic sensitivity tests were carried on MHA plates by Kirby-Bauer disk diffusion method using HiMedia ready to use antibiotic discs showed a different zone of inhibition (Table 2). Characterization of the isolates as susceptible, intermediately resistant, or resistant was based on the size of inhibition zones around each disc as per the manufacturer's instructions, which matched the interpretive criteria as per CLSI guidelines (Table 2) (CLSI, 2007) [8].

DISCUSSION

Food contamination continues to be a worldwide problem. Recent development in food production and processing techniques and the subsequent changing trends in food consumption have resulted in the emergence of new hazards. Consumption of untreated water and uncooked seafood in summer is another epidemiologic evidence of *V. cholerae* transmission [8]. Therefore; this study was conducted to isolate, to identify and antibiotic susceptibility evaluation of *V. cholerae* in different fish species. *V. cholerae* the causative agent of cholera was first described by Robert Koch in 1883. *Vibrio* organisms are free living widely distributed highly motile Gram-negative curved or comma-shaped rods with a single polar flagellum, and most species are oxidase positive [9]. *V. cholerae* is spread by eating food or drinking water that has been contaminated with cholera bacteria. Contamination usually occurs when human feces from a person who has the disease seeps into a community water supply [10]. In different studies, the incidence of *V. cholerae* sample collection was done from different localities to give a good chance for isolation of different bacteria and for determination greater incidence in different food materials, in our study the sample collection was done in local market targeting different

Table 1: Biochemical tests performed

Tests	S1	S2	S3	S4
Gram staining	-	-	-	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
Citrate utilization	+	+	+	+
Nitrate reduction	+	+	+	+
Indole test	+	+	+	+
Methyl red	+	+	+	+

Table 2: Antibiotics susceptibility profile of isolates

Antibiotics	Zone of inhibition (mm)				Interpretive chart according to NCCLS			
	S1	S2	S3	S4	S1	S2	S3	S4
Penicillin G	17	15	13	18	S	S	S	S
Streptomycin	17	18	18	15	S	S	I	I
Chloramphenicol	25	15	25	23	S	I	S	S
Ampicillin	35	32	30	33	S	S	S	S
Amoxicillin	0	0	0	0	R	R	R	R
Tetracycline	30	25	25	27	S	S	S	S
Ciprofloxacin	24	24	25	25	S	S	S	S
Co-trimoxazole	25	26	30	27	S	S	S	S
Nitrofurantoin	6	7	0	0	R	R	R	R
Norfloxacin	27	28	25	23	S	S	S	S

S1, 2, 3, 4: Isolate one, two, three, and four; S: Susceptible, I: Intermediate, R: Resistance

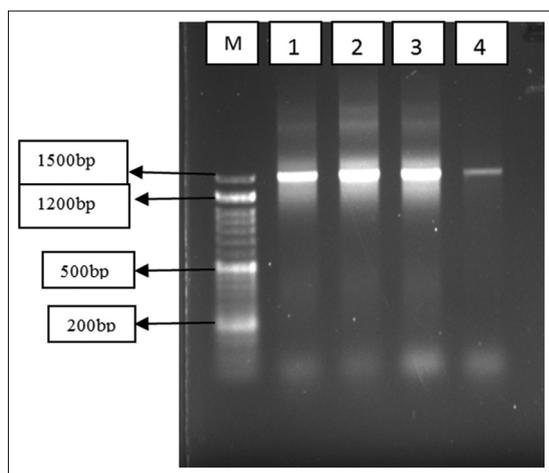


Fig. 1: Agarose gel electrophoresis of polymerase chain reaction products amplified with 16S rRNA primer from the genomic DNA of *Vibrio cholerae* isolates

marine fish species available, in this study the selected fish species show the presence of targeted bacteria this show the contamination of water also, here the prevalence of *V. cholerae* is high. Biochemical tests performed revealed phenotypic similarities of the two species observed in the results of oxidase, TSI, catalase, and methyl red Voges-Proskauer tests [11] asserts that the tests which have been applied in this study were able to efficiently differentiate these species. Thus, for detection of the species of the isolates, the conventional biochemical tests showed the low efficiency. Molecular identification becomes a great tool in the area of clinical diagnosis; PCR-based detection targets the specific region of DNA, for identification of bacterial strains. Furthermore, PCR facilitates the identification of the strains that are viable but non-culturable [12] in this study 16S rRNA gene was amplified and showed great results. It is less labor intensive and much faster than conventional methods, and that is the reason of its increasing application among investigators [13]. The antibiogram profile revealed that isolates, all isolates showed multi-drug resistant to amoxicillin and nitrofurantoin. And they are susceptible to the remaining antibiotics used in this study which is commonly used to treat cholera infection. Mukhopadhyay *et al.* [14] reported the ineffectiveness of co-trimoxazole and furazolidone to treat patients with *V. cholerae* O1 infection and appearance of nalidixic acid resistance among O1 strains from Calcutta patients [15], isolated and reported tetracycline resistant strains in Kolkata in the year 2005. There is an agreement between the results that show high individual and multiple antibiotics resistance among all examined *Vibrio* strains, and other researchers [16,17]. Antibiotics provide the main basis for the therapy of microbial infections. However, the high genetic variability of microorganisms enables them to rapidly evade the action of antibiotics by developing antibiotic resistance [18]. One study revealed that all *Vibrio* strains were found to harbor antibiotics resistant genes and showed resistances to ampicillin, furazolidone, nalidixic acid, streptomycin, trimethoprim-sulfamethoxazole, and trimethoprim [19]. Thungapathra *et al.* indicated that in a total number of 94 isolates of *V. cholera*, 43 strains contained R-plasmids and exhibited resistances to ampicillin, neomycin, tetracycline, gentamicin, streptomycin, sulfonamide, furazolidone, and chloramphenicol [20]. The isolates are not much resistant consider to the other well-reported cases of *Vibrio* outbreaks. Hence, there is an urgent need for the discovery of new and novel antimicrobial drugs to effectively eradicate the diseases producing microorganisms [21].

CONCLUSION

This is the study that describes the occurrence, molecular characterization and antibiotic susceptibility pattern of *V. cholerae* isolated in marine fish species. Molecular methods could be rapid and concise methods for the detection of foodborne bacteria. This

study revealed the development of multidrug resistance among *Vibrio* strains in this region. High measures need a continuous vigilance on the changing trend in the antibiotic susceptibility pattern of *V. cholerae* which is due to environmental factors and widespread use of antibiotics. Finally, the results of this study indicated possible risks to consumers of marine fishes in the region that demand action to address this public health concern.

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