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Evaluation of Polymerase Chain Reaction for the detection of *Vibrio cholerae* in Contaminants

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ABSTRACT

A total of 245 samples (35 each of water, fish, crab, shrimp, meat, milk and clinical stool samples) collected from various sources were subjected to Polymerase Chain Reaction (PCR) and cultural methods for the presence of Vibrio cholerae. Eighty samples (19 water,16 fish, 20 crab, 6 shrimp, 4 meat, 3 milk, and 12 clinical stool samples) were positive by PCR targeting ompW gene, whereas only 59 samples (12 water, 13 fish, 16 crab, 5 shrimp, 3 meat, 2 milk and 8 clinical stool samples) were positive by cultural methods. Enrichment with Alkaline Peptone Water (APW) gave good results compared to Salt Polymixin Broth (SPB) with polymyxin B by both PCR and cultural methods. The minimum detection level with pure V.cholerae culture was 2.5cfu/ml with Alkaline Peptone Water broth after 8 hrs of incubation. The results of this study suggest that PCR could be an excellent tool for detection of Vibrio cholerae in aquatic, livestock foods and stool samples.

Keywords: Vibrio cholerae, PCR, Prevalance, Cultural method.

INTRODUCTION

Vibrio cholerae is important water borne facultative human pathogen of worldwide gastrointestinal disease significance. Cholera is a life threatening diarrhoeal disease, still kills thousands annually and remains one of the few bacterial diseases known for its pandemicity [1]. During 2007, a cholera outbreak hit many parts of Orissa in the wake of massive flooding following South Asia's worst monsoon season in living memory, wherein thousands of tribal people affected and hundreds of deaths occurred [2].

Cholera has been recognized as one of the emerging and re-emerging infections in developing countries [3] and it is classified as Category B bioterrorism by Centres for Disease Control and Prevention [4]. V. cholerae is associated with plankton and other aquatic organisms [5]. Sea foods including molluscan shellfish, crustaceans and finfish are most often incriminated in food borne cholera cases in many countries [6]. Therefore ingestion of raw or undercooked seafood such as shrimp and drinking water contaminated with *V.cholerae* are risk factors in humans [7]. Etiological studies on acute diarrhoeal diseases in gangetic plain areas have shown that gastroenteritis caused by V.cholerae ranks first in terms of incidence followed by V.parahaemolyticus in India [8] and other developing countries [9].

The conventional isolation procedures includes growth in enrichment broth (Alkaline Peptone Water) followed by plating on selective media i.e., Thiosulfate Citrate Bile salt Sucrose Agar (TCBS). The process, however, is laborious and time consuming. Further, close relatedness among V.cholerae and certain other members of the Vibrio spp (e.g. V.mimicus) or Aeromonas spp. with respect to their biochemical properties has often made unambiguous identification of the organism quite difficult [10]. The PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection and identification of specific pathogenic bacteria from different food materials [11, 12]. Restriction fragment length polymorphism analysis and nucleotide sequence data revealed that the ompW sequence is highly conserved among V. cholerae strains belonging to different biotypes and/or serogroups. All of these results suggest that the ompW gene can be targeted for the species-specific identification of V. cholerae strains [13]. Hence, in this study an attempt has been made to screen different contaminants for the presence of V. cholera by both PCR and cultural methods.

MATERIALS AND METHODS

Thirty five each of fish, crab and shrimp samples (50g) were collected from local fish markets. Milk samples (50ml) were collected from college dairy experimental station, local dairy farms, local vendors and markets. Meat samples (50g) were collected from local and Greater Hyderabad Municipal Corporation markets at Chengicherla. Water samples (50ml) were collected from hostels, livestock farms and local farms and clinical stool samples were collected from diarrhoeal patients admitted to various government hospitals in Hyderabad.

Samples were enriched in 90 ml of Alkaline Peptone Water (APW) and Salt Polymixin Broth (SPB) and incubated at 37°C for 8 -18 hours. The broth cultures were spread plated onto Thiosulfate Citrate Bile Salt Sucrose agar (TCBS) and incubated at 37°C for 24 hours for isolation of V.cholerae. The sucrose fermenting yellow colored colonies were taken for further confirmation by biochemical tests like IMViC (Indole, Methyl Red, Voges Proskeur and citrate utilization) tests, nitrate test, catalase test and urease test. Isolates produced acid slant with acid butt with no gas production on Triple Sugar Iron (TSI) agar and oxidase, sucrose, indole, ornithine, arginine and lysine positive and motile were conformed as V. cholerae.

All the enriched samples were subjected to PCR analysis for the presence of V.cholerae using primer specific to outer membrane protein gene (ompW) (Table I). V. cholerae strain, obtained from Microbial Culture Type Cell Culture (MTCC) Chandigarh, was used as known positive strain in PCR analysis. 1.5ml of the enriched broths was taken into eppendorf tubes and bacteria

were pelleted by centrifugation at 8000 rpm for 5 min. To the pellet, 50µl of molecular grade water was added and incubated at 100°C for 10 min and snap chilled to release DNA. Then centrifuged at 13,000 rpm for 5 min and the supernatants were used as DNA templates for PCR analysis.

Table I. Oligonucleotide primer used in the study

Primer	Target gene	Primer sequence	Fragment size (bp)	Reference
ompW- F	ompW	CAC CAA GAA GGT GAC TTT ATT GTG	304	[14]
ompW- R	ompW	GGT TTG TCG AAT TAG CTT CACC	304	[14]

Bacterial DNA amplification was done in 20 µl reaction mixture containing 2 µl of 10X Taq DNA polymerase buffer (containing 100mM Tris with pH 9.0, 50mM KCl, 15mM MgCl2 and 1% Triton X-100), 2 µl of 10mM of dNTP mix, 0.9U of Taq DNA Polymerase (Genei), 2 µl each of 10 Pmoles/ µl of forward and reverse primers, 5 µl of crude bacterial lysate and made to 20 µl using molecular grade water. Amplification was done in thermal cycler following standardized conditions (Table II). The amplified DNA fragments were resolved by agarose gel electrophoresis, stained with ethidium bromide (0.5 µg/ml) and visualized with an UV transilluminator.

Table II. Cycling conditions used for the ompW gene primers

S.No.	Step	ompW
1.	Initial denaturation	94°C/ 5 min
2.	Final denaturation	94°C/ 1 min
3.	Annealing	55°C/ 1 min
4.	Initial extension	72°C/ 1 min
5.	Final extension	72°C/ 7 min
6.	Hold	4°C

Sensitivity of PCR method for V.cholerae was reassessed with the help of spiking studies using homogenized fish inoculated at the rate of 250 cfu, 25 cfu, 2.5 cfu and 0.25 cfu of V.cholerae (MTCC, Chandigarh) strain per 10g of fish and transferred to two different enrichment media i.e. APW and TSB. The PCR and cultural testing were carried after 8hr and also at 18 hr of incubation at 37°C.

RESULTS AND DISCUSSION

The protocols followed in this study for DNA extraction are inexpensive, do not leave any inhibitory residue in the sample and produced stable DNA extracts. This procedure has been used previously for the detection of V.cholerae in food materials [12]. The detection level of V.cholerae with APW broth was 2.5cfu, whereas it was 25 cfu for SPB broth using PCR. Spiking studies also reconfirmed suitability of APW broth over the SPB as enrichment media for V.cholerae, as it multiplies rapidly in the former [15]. Enrichment incubation time of 8hr was found optimum for getting specific V.cholerae isolates as vibrios may be gradually overgrown by other enteric bacteria after 8hrs of incubation [16]. Enrichment in APW followed by culturing on

TCBS agar resulted in faster multiplication of V. cholerae isolates than other enteric bacteria, which gave the best results as compared to direct plating on agar [17]. Further, isolation and differentiation of V.cholerae made easy with TCBS culturing media [18] as it gave yellow colored colonies with sucrose fermenting Vibrios (V.cholerae) and green colonies with non sucrose fermenting vibrios (V.parahaemolyticus).

Among the tested water samples, 63.16% (19 out of 35) were positive by PCR, where as the cultural method was able to detect V. cholerae in 34.29% (12 out of 35) of samples (Table III, Fig 1 and 2) and this clearly indicates the sensitivity of PCR over cultural methods in detection. V. cholera.

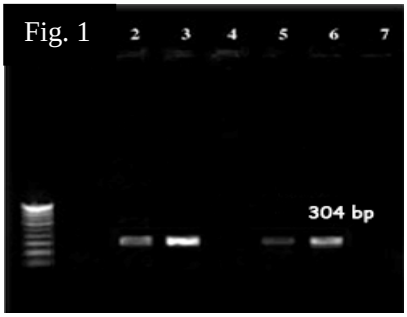
Table III Cultural and PCR results of different natural samples for V.cholerae

Type of sample	No. of samples	Positive result for V. cholerae ompW				
		Cultural method	%	PCR assay	%	% of cultural method compared to PCR
Water	35	12	34.29	19	54.29	63.16
Fish	35	13	37.14	16	45.71	81.25
Crab	35	16	45.71	20	57.14	80.00
Shrimp	35	5	14.28	6	17.1	83.50
Meat	35	3	8.57	4	11.43	74.98
Milk	35	2	5.71	3	8.57	66.62
Clinical stool samples	35	8	22.85	12	34.29	66.63
Total	245	59	24.08	80	32.65	73.75

The high incidence of V. cholerae in water samples indicating the natural inhabitant of the organism in aquatic environments, hence water plays a central role in the transmission of cholera. In order to circumvent potential cholera threats, it is paramount to determine the prevalence of V. cholerae in aquatic environments. Such surveillance systems require methods that are reliable, reproducible and rapid [19, 20].

The incidence of this organism in milk samples was found to be very low in the present study. Enrichment of the samples didn't show any significant increase in the isolation of the organism. [21] also reported almost similar incidence of V.cholerae in milk. The lower incidence of V.cholerae in milk samples might be due to management of good hygienic practices during milking, where water is the main source of contamination [22].

In most of the fish samples, the bacteria were isolated without the use of any enrichment methods, signifying that V. cholerae is abundant in fish species. Copepods and chironomids, both natural reservoirs of V. cholerae, are abundant in fresh and marine water ecosystems and are consumed by fish. Sixteen samples (45.7%) were positive by PCR, where as the cultural method was able to detect V. cholerae in 13 samples (37.1%) indicating superiority of PCR over cultural methods. [23] reported high incidence of V. cholerae (60%) in marine fish and fresh water fish (50%) using PCR technique. Similarly PCR detected high incidence of V.cholerae contamination in seafood samples like crabs (80%) and shrimps (83.5%). Further, enrichment with APW before amplification increased the detection limit of 105 fold and detected as few as 100 bacteria per 10 g, which is well below the usual infectious dose by oral ingestion [24].



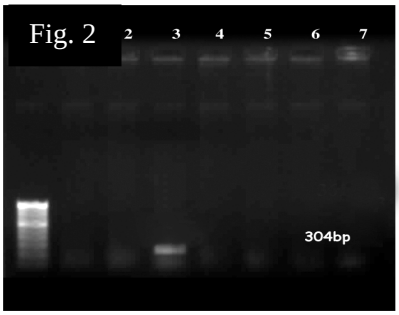


Fig 1. Agarose gel electrophoresis of PCR amplified product ompW gene of V.cholerae in water sample (Lane M: 100 bp DNA Ladder, Lane 2,3,5,6: water samples showing positive result).

Fig 2. Agarose gel electrophoresis of PCR amplified product ompW gene of V.cholerae in milk sample (Lane M: 100bp DNA Ladder; Lane 3: milk sample showing positive result showing positive results).

V.cholerae was detected in 4 meat and 12 clinical stool samples by PCR targeting ompW gene, whereas cultural method detected only in 3 meat and 8 clinical stool samples. A total of 80 samples were found positive by PCR, where as cultural methods were able to detect only 59 samples as positive in the total collected samples (245). The percent of positive results by cultural method compared to PCR assay was only 73.75. Failure of the cultural methods in detection of the V.cholerae organism in some of the samples could be due to presence of the organism in viable but non-culturable state (VBNC), where they retain viability and infective potential [25] over years [26] but the conventional culture methods failed to detect [27, 28].

CONCLUSION

Alkaline Peptone Water (APW) broth is the best media for growth of V. cholerae. The minimum detection level required for detection of V.cholerae in contaminants was a minimum of 2.5cfu/ml by PCR. Further, TCBS culturing media helps in isolation and differentiation of V. cholerae from V. parahaemolyticus. The results of this study suggest that PCR could be an excellent tool for detection of Vibrio cholerae in aquatic, livestock foods and stool samples that act as contaminants for the spread of cholera disease in humans.

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