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ORIGINAL RESEARCH ARTICLE

Identification of *Vibrio parahaemolyticus* Isolates by PCR Targeted to the *toxR* Gene

P. Elamparithi* and N.Ramanathan

Department of Agricultural Microbiology, Faculty of Agriculture,Annamalai University, Annamalai Nagar – 608 002, Tamil Nadu, India

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ABSTRACT

Vibrio parahaemolyticus is a major cause of food borne illness such as gastroenteritis in human through consumption of undercooked seafood or wounds exposed to marine animals or warm coastal waters especially in Southeast Asian. This is because a short warm period is sufficient for *Vibrio parahaemolyticus* which has short generation time to grow until infectious levels (10^6 organisms). There are many methods used in the detection of *Vibrio parahaemolyticus*. The standard selective medium method used for *Vibrio* is Thiosulfate citrate bile salt sucrose agar (TCBS). *Vibrio parahaemolyticus* colonies are green or blue green on the agar due to sucrose fermentation. The latest technique would be PCR that can be used for detection of *Vibrio parahaemolyticus* in various samples including seafoods or other samples, and this method is faster, easier and more reliable. This technique can be applied because of the presence of *toxR* gene that appears to be well conserved among *Vibrio parahaemolyticus.* This gene can be used to develop a PCR method for identification of *Vibrio parahaemolyticus.* In this study, the fish sample (Sangara– Red snapper, *Lutjanus compechanus*) was collected from costal belt of Cuddalore district. When the sample was streaked on the TCBS agar, Green colour *Vibrio parahaemolyticus* colony was observed and was they were grown on selective medium, TCBS agar. Specific-PCR for *toxR* gene detection gave positive result in which a band with 368 bp size appeared on gel confirmed the presence of *Vibrio parahaemolyticus.*

Keywords: - *Vibrio parahaemolyticus*, TCBS, *toxR* –gene and PCR.

1. INTRODUCTION

Vibrio parahaemolyticus is a bacterium in the same family as those that cause cholera. It lives in brackish saltwater and causes gastrointestinal illness in humans. It is a halophile, or salt-requiring organism. Most people become infected by eating raw or undercooked shellfish, particularly oyster and cockles. At least 12 *Vibrio* spp. are classified as pathogenic strains and become major factor for food-borne diseases. *Vibrio parahaemolyticus* caused about 25% of total food-borne diseases in comparison to other *Vibrio* species[1]. Three species of *Vibrio* (from 28 species) that are often associated with *Vibrio parahaemolyticus* in aquatic environmental and seafood are *Vibrio vulnificus*, *Vibrio alginolyticus* and *Vibrio cholerae.*

Vibrio parahaemolyticus is a major cause of food-borne illness such as gastroenteritis in human through consumption of undercooked seafood or wounds exposed to marine animals or warm coastal waters especially in Southeast Asian [2]. This is because a short warm period (temperature range from 10^oC- 43^oC, optimum is 37^oC) is sufficient for *Vibrio parahaemolyticus* which has short generation time (8-9 minutes) to grow until infectious levels (10^6 organisms) [3]. There are many methods used in the detection of *Vibrio parahaemolyticus*. The standard selective medium method used for *Vibrio* is Thiosulfate citrate bile salt sucrose agar (TCBS). *Vibrio parahaemolyticus* colonies are green or blue green on the agar due to sucrose fermentation [4].The latest technique would be PCR that can be used for detection of *Vibrio parahaemolyticus* in various samples including seafoods or other samples, and this method is faster, easier and more reliable. This technique can be applied because of the presence of *toxR* gene (was first discovered as the regulatory gene of the toxin operon), that appears to be well conserved among *Vibrio parahaemolyticus.* This gene can be used to develop a PCR method for identification of *Vibrio parahaemolyticus* [5]. Other than *toxR* gene,

***Corresponding Author:** P.Elamparithi**, Email:** bioelam@gmail.com

gyrB gene also can be used for detection using PCR because this gene is also found to be well conserved among *Vibrio parahaemolyticus* isolates [6]. The objective of this study is to detect the presence of *Vibrio parahaemolyticus* isolates on the selective medium, TCBS. This test was further confirmed by specific-PCR to detect the presence of toxin operon (*toxR)* gene.

2. MATERIALS AND METHODS

2.1.Sampling and processing of samples

The fish sample containing 25g of gut potion (Sangara- Red snapper *Lutjanus compechanus*) was homogenized in 225 ml Alkaline Peptone Water with the addition of 3% NaCl. The sample was allowed to settle down in Alkaline Peptone Water (hereinafter called APW enrichment broth) at room temperature for 15 min. Then, they were incubated at 37^0C for 24 hrs. After that, 1 ml portion of each of the broth culture was transferred into a sterile bottle containing 1 ml salt polymixin broth and incubated at 35- 37^0C overnight.

2.2.Identification on TCBS agar

From the salt polymixin broth culture, a loop full of culture was streaked on the TCBS agar surface and later sealed with parafilm before incubated for overnight. Pure Green colonies were selected and were streaked onto a new TCBS agar medium surface for purity and then were grown in Luria-Bertani (LB) broth and later on LB agar (as a stock). Confirmation by specific-PCR (detection of *toxR* gene).The selected colonies from TCBS agar were tested using PCR method to detect the presence of *toxR* gene which appeared highly conserved in *Vibrio parahaemolyticus* isolates [5].

2.3.Genomic DNA extraction

Test culture was grown in 3.0 ml Luria-Bertani broth at 37^0C for overnight. The cell suspension was transferred to 1.5 ml microfuge tube and was centrifuged at 10,000 rpm, 25^0C for 2 min. The supernatant was discarded and 1 ml of sterile distilled water was added and vortexed to mix. The cell suspension was then centrifuged at 10,000 rpm, 25^0C for 5 min. The supernatant was discarded and again 1 ml of sterile distilled water was added. After vortex, the cell suspension was incubated at 60^0C for 10 min in water bath. Immediately the tube was immersed into ice and kept for 10 min and after a short spin (10,000 rpm for 15 sec), the supernatant was transferred to a new tube. The supernatant contained genomic DNA and is ready to be used as a template for *toxR* genes detection in *Vibrio parahaemolyticus.* Specific primers (R: 5'-GTCTTCTGACGCAATCGTTG - 3' and F: 5' – ATACGAGTGGTTGCTGTCATG – 3') correlated to *Vibrio parahaemolyticus toxR* region with 50% GC contents [5]. The primer was obtained from Bio-serve India Pvt. Ltd. Hyderabad, India.

2.4. PCR-Protocol

PCR (Perkin Elmer) was carried out using 0.5 ml microfuge tubes. The total volume of reaction mixtures was 20.0 µl which consisted of 14.3 µl sterile distilled water, 2.0 µl 10x PCR buffer with MgCl2, 1.6 µl 25 mM deoxyribonucleotide phosphate, 0.8 µl of each primer, 0.1 µl of 0.5 units *Taq* DNA and 1.2 µl template DNA. The cycling conditions were as follows; predenaturation at 96°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min at the end of 35 cycles.

2.5. Agarose Gel Electrophoresis

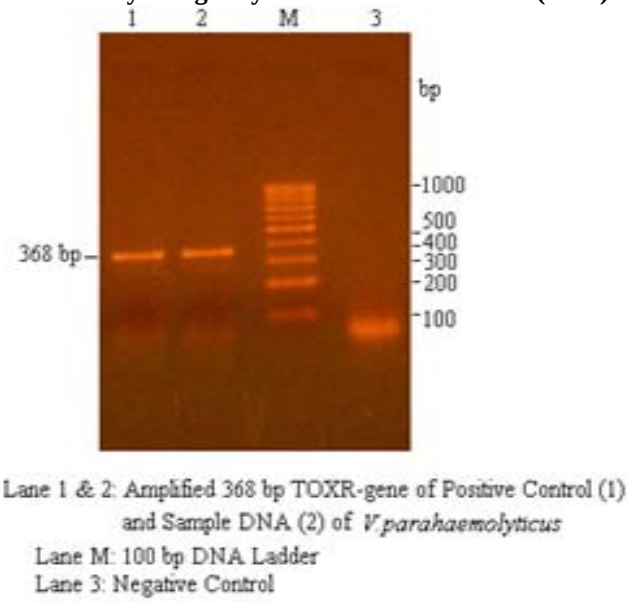
The PCR products were run on 1.2% agarose gel (Sigma) in 1x Tris-Borate- EDTA (TBE). 15 – 20 µl PCR products were loaded into sample wells and the voltage used was 100 volt for 1 h. The gel was then stained in Ethidium bromide (0.5 µg/ml) solution for 1 min and destained in distilled water for 30 min. Then the gel was visualized and photographed under UV transilluminator.

3. RESULTS AND DISCUSSION

The fish sample (Sangara– Red snapper, *Lutjanus compechanus*) was collected from costal belt of Cuddalore district, tested on the TCBS agar for the detection of *Vibrio parahaemolyticus*. When the samples were streaked on the TCBS agar two types of colonies were observed which were, Green (*Vibrio parahaemolyticus*), and Yellow (*Vibrio vulnificus*). Various reports state that three species of *Vibrio* usually associated with *Vibrio. parahaemolyticus* in aquatic environmental and seafood were *Vibrio vulnificus*, *Vibrio alginolyticus* and *Vibrio cholerae.* The selection of the isolates were based on the strong colony appeared with the pure green in colour. In TCBS agar medium, which contains a chromogenic substrate as substrates for beta galactosidase, instead of sugar fermentation (sucrose) for the detection of *Vibrio parahaemolyticus* by Green colony [6]. Identifying *Vibrio parahaemolyticus* strains through PCR based method which targets the conserved region of *Vibrio parahaemolyticus* such as *gyrB* and *toxR* gene is more efficient, reliable and faster compare to biochemical test. After the identification on the TCBS agar, the PCR assay for *toxR* detection was performed to confirm the presence of *Vibrio parahaemolyticus* [5].

Dileep *et al.* (2003)[7] and Sujeewa *et al.* (2009)[8] reported that the *toxR* gene fragment (~368 bp) which is specific for *Vibrio parahaemolyticus* was successfully amplified and results are presented in (**Fig 1**). In this study, the primer used for the amplification of *toxR* gene is specific for *Vibrio parahaemolyticus* on 1.2 % agarose (Lane-1) as the positive control,(Lane-2) as the sample DNA of *Vibrio parahaemolyticus*, (Lane-M) as the 100 bp DNA ladder and (Lane-3) as the negative control.

Fig 1: Detection of *toxR*-gene of *Vibrio parahaemolyticus* by using Polymerase Chain Reaction (PCR)



Lane 1 & 2: Amplified 368 bp TOXR-gene of Positive Control (1) and Sample DNA (2) of *V.parahaemolyticus*
Lane M: 100 bp DNA Ladder
Lane 3: Negative Control

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