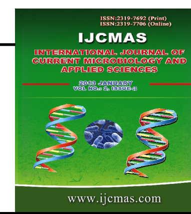
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 3 (2013) pp. 6–10**
http://www.ijcmas.com



Original Research Article

Rapid identification of *Vibrio harveyi* isolates in *Panulirus homarus*

V.A.Leslie[1], A. Margaret Muthu Rathinam[2] and Anusha Balasingh[3*]

[1]Madras Research Centre of Central Marine Fisheries Research Institute, Chennai 600 028, India, Email: lesliecmfri@gmail.com
[2]Madras Research Centre of Central Marine Fisheries Research Institute, Chennai 600 028, India, Email: amrathinam@reddifmail.com
[3]Department of Microbiology, Ethiraj College for Women, Chennai 600 006, India
**Corresponding author:* anusha_274@yahoo.com

Keywords

PCR;
Tail fan necrosis;
Vibrio harveyi;
Panulirus homarus;
Live lobsters

A B S T R A C T

Vibrio is the most common genera associated with Crustaceans often causing significant economic losses. Many Vibrio species are pathogenic to human and have been implicated in the food borne disease. Tail fan necrosis is of recognized constraint in lobsters live holding industry because of reduction in value of affected lobsters. Aquaculture has an important role in the development of many national economics and play a key role in rural development. As the expansion of aquaculture product, there is a concern over the impact of aquaculture on the environmental sustainability and also over the requirements on quality and food safety for consumers and regulators. For this reason, it is a need to improve aquaculture technology and management system to address the need for the eco-friendly production process and food safety concerns in sustainability of national aquaculture. In the present study a total of 11 luminescent bacteria were isolated from the infected Indian spiny lobster *Panulirus homarus*. Many of the lobsters in the holding facilities developed a range of exoskeleton and tail lesions. The splitting and crackling of the chitin could have allowed the entrance of the bacteria such as *Vibrio harveyi*, commonly present in water. Predisposing factor could have included handling of the animals, the stress of holding resulting in decreased immunocompetence, injury from fighting or abrasions from the cage wire and elevated water temperatures during the period of holding, *V. harveyi* is an important pathogen and is extremely difficult to identify because it is phenotypically diverse. Hence PCR technique was employed using 16S rDNA sequences to reduce the duration of identification of this species, a valuable tool for a rapid and accurate detection and hence earlier treatment can be administered which may increase the survival rate from vibriosis.

Introduction

Pathogenic marine vibrios such as *Vibrio harveyi*, *Vibrio vulnificus* and *Vibrio parahaemolyticus* are significant human pathogens. Some strains of vibrios are also known to cause diseases (eg.) shell in various crustaceans such as lobster and prawn. Enteric diseases associated with these pathogens are caused by ingestion of contaminated seafood. Consequently, there is a significant industry interest in

determining potential risk posed by the contamination of these sea foods with vibrios when they are grown in captivity.

Vibrio harveyi is a luminous bacteria found abundantly in marine and estuarial habitats (Hastings *et al*., 1981; Jiravanichpaisal *et al.,* 1994). Some strains of this bacterium are highly pathogenic to aquatic fauna, especially invertebrates and other strains may be considered opportunistic pathogens.

In the present study, the Indian spiny lobster *Panulirus homarus* affected by tail diseases were examined. These lobsters were kept captive in holding facilities to maximize harvest returns. The infected lobster developed necrotic lesions on the uropods of the tail. These lesions increase in size and severity and can cause significant disfigurement of the tail fan, often accompanied by inflammation.

In severe cases, tail fan necrosis can lead to loss of most of the uropod. The significant erosion of melanisation accompanying this syndrome makes affected lobsters unsalable. The tail fan necrosis lesions are usually characterized by the colonization of bacterial micro colonies dominated by *Vibrio* spp.

The chitinous exoskeleton of Indian spiny lobsters is an effective barrier that prevents the entry of infectious agents as well as providing muscle anchorage and protecting underlying soft tissue. The first barrier presented by the exoskeleton against invasion is the very thin proteolipid epicuticular membrane. Beneath this layer is the calcified exocuticle. This is very difficult to penetrate, even for disease agents secreting extracellular chitinase. By contrast the soft non calcified endocuticle is easily penetrated by such agents.

Lobster health is also influenced by a range of factors, one of the most important of which is stress. Stress responses are normal physiological reaction to changes in environmental conditions. These conditions include a wide range of factor such as water quality parameters (oxygen levels, pH, salinity, temperature, presence of toxins) physical factors (handling, injury, air exposure), behavioral interactions and nutrient availability. Hence holding live lobsters for extended periods for fattening may pose problems experienced with mortalities, shell and tail disease etc.,

Generally only *V. harveyi* can cause the mass mortalities resulting from vibriosis. Phenotypically *V. harveyi* is highly heterogeneous and therefore extremely difficult to identify using conventional bacteriological tests or kits relying upon biochemical reactions (Vandenberghe *et al.,* 2003). Complex keys using very high numbers of phenotypic characteristics have been reported. Castro *et al.,* (2002) used 94 tests to differentiate halophilic vibrios, while there are reports ranging upto 109 tests. Such high numbers of tests are not feasible for routine diagnostic laboratories because of the time and costs involved. Moreover, this *V. harveyi* heterogeneity is further confounded by evidence that *V. harveyi* contain mobile genetic elements such as plasmids (Harris, 1993) and bacteriophages (Oakey and Owens, 2000), some of which carry phenotypic characteristics (Munro *et al.,* 2003). In addition, some genetic heterogencity of the species has been suggested by Caccamo *et al.,* (1999) based upon random amplified polymorphic DNA-PCR analysis of a *V. harveyi.*

It has become apparent that a more rapid and definitive method is required for the confirmation of identify of isolates of *V. harveyi.* Such a method would be

valuable as a research tool and a diagnostic tool for the identification of *V. harveyi* as the aetiological agent of vibriosis.

The phenotypic and genetic heterogencity and the presence of mobile genetic elements of *V. harveyi* mean that a species-specific marker common to all isolates would be extremely difficult, if not impossible, to locate. However, the gene for 16S ribosomal RNA (16S rDNA) is highly conserved in eubacteria are essential to the viability of the cell. This gene, of approx. 1500bp, contains regions of high nucleotide sequence conservation and small regions of variation. These small regions of variation are commonly utilized by biotechnologists to design priming sites for diagnostic PCRs for bacteria that are heterogeneous, rather than using conventional bacteriological testing.

The purpose of the described work is to investigate the design and use of a PCR to assist with the identification of *V. harveyi* based upon priming sites within the 16S rDNA sequence, hence reducing the number of biochemical tests, and time required.

Materials and Methods

Live lobsters of the size ranging from 45mm to 50 mm were obtained from a Kasimedu landing centre at Chennai, South India and brought to the Kovalam laboratory, Chennai, Tamilnadu, India. They were acclimatized to 28±1 ºC temperature and a salinity of 35 ppt for 5 days.

The lobsters were then stocked in FRP tanks according to their sizes and were fed with clams for fattening. The study was conducted for a period of 150 days. During this period the water in the tank and the animals were acclimatized by luminescent vibrios. These pathogenic vibrios infected the tissues, gills, gut and exoskeleton. Hence isolation and few preliminary and biochemical characterization tests were performed which were not adequate and authentic to differentiate the vibrios to species level. Hence a more rapid and authentic molecular tool for the diagnosis luminescent bacteria was designed using suitable primers to run PCR technique.

DNA extraction

Total genomic DNA was extracted from 12 different isolates of *Vibrio harveyi* and 1 MTCC strain 3438 grown in sea water agar. Extracted DNA was stored at -20 ºC which served as a template DNA.

16S rDNA sequence determination

16S rDNA sequence of 14 strains of *V. harveyi* were amplified from DNA extracts using primer sequences developed by Medox Biotech India Pvt. Ltd., PCR was carried out using Forward primer and Reverse primer sequences as 5¹-AAC GAG TTA TCT GAA CCT TC -3¹ and 5¹-GCA GCT ATT AAC TAC ACT ACC -3¹. The sequence of forward primer concentrate for a volume of 100 p mol/µl was found to be 342µl with a molecular weight of 6076g/mol. The Tm value was calculated as 47.7 ºC and GC content was found to be 40% and purified by desalting technique. Whereas in case of Reverse primer for a volume of 100 p mol/ µl the concentration was found to be 349 µl and the molecular weight to be 6334.2g/mol. The Tm values were calculated as 50.5ºC, GC content as 43% and were purified by desalting technique.

The PCR technique was carried out using PCR kit KT 44 provided by Bangalore Genei. The reagents to be added to the PCR tube was proceeded in the following order

Sterile water	38 µl
10 x Assay Buffer	5 µl
10mM d NTP Mix	1 µl
Template DNA (100ng/ µl)	1 µl
Forward primer(100ng/ µl)	1 µl
Reverse primer(100ng/ µl)	1 µl
Taq DNA polymerase (3U/ µl	1 µl

Total reaction volume was accounted as 50 µl, all the above contents were mixed gently and the reaction mix was layered with 50 µl, of mineral oil to prevent evaporation. (Mineral oil is not needed if the thermocycle is equipped with a heated lid)

The amplication in a thermocycler was carried out for 30 cycles using the following reaction condition. The initial denaturation temperature was set as 94ºC for 1 minute and the second denaturation temperature at 94 ºC for3 seconds. The annealing step was carried out at 48ºC for 30 seconds with extension reaction at 72ºC for 1 minute and final extension carried out for 2 minutes at 72ºC. Following PCR amplification 5 µl of Gel loading buffer was added to the PCR tubes.

The mixture was mixed thoroughly and 15 µl of the reaction mixed was carefully pippetted out and loaded on to 1.5% agarose gel. The samples were run at 100 volts for 1-2 hours till the tracking dye reaches 3/4th of the length of the gel using agarose gel electrophoresis technique. The gel was then visualized under UV transilluminator.

The amplified bands were compared with a 100bp ladder to determine the size of the fragment.

Results and Discussion

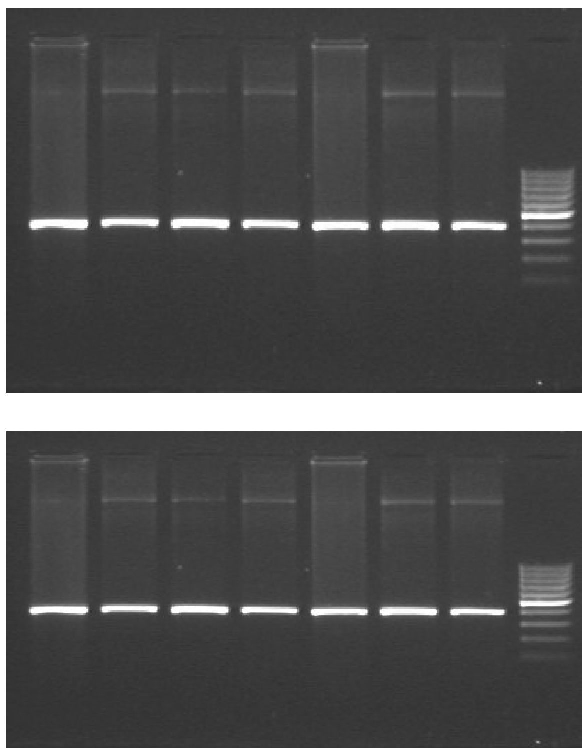
The PCR technique was found to assist the confirmation of identity of 14 different isolates of suspected *Vibrio harveyi* from infected lobsters. The time factor indicates that affected lobsters can be diagnosed in stipulated timings with accurate results than previously spend on biochemical morphological testings.

As observed on agarose gel PCR amplification of the template using specific primers results in a specific product of a particular length. All the isolates have a specific product yield of 400 bp as observed. Hence PCR was carried out with 100 nanogram of template, having approximately about 1000 copies of the target sequence (Fig.1). Following PCR, the product yield is in microgram quantity, which is approximately a million copies of the target sequence, high lighting the fact that PCR is a very sensitive technique. Due to phenotypic similarities and genome plasticity, traditional identification and typing methods are not always able to resolve *V. harveyi* from closely related species. The paper provides an overview and evaluation of molecular method currently used to identify and type *V. harveyi* during epidemiological outbreaks and present prospects and challenges for the early detection of *V. harveyi* in complex samples. The rapid expansion of the lobster aquaculture industry over the least few decades has provided many countries with high revenues.

Concomitant with the growth of the lobster culture industry is the occurrence of many bacterial and viral diseases. This often leads to significant financial losses for many lobster farmers. One of the important ways to prevent bacterial epidemic in lobster fattening system is to use filtered sea water. Physico-chemical parameters such as temperature, salinity and pH of the water samples have to be maintained in order to prevent bacterial invasion. Hence a successful method of screening the most

common luminous pathogenic bacteria causing tail fan necrosis is used in the present study.

Fig 1. PCR amplified products from 14 different isolate of *V. harveyi*



Lane 1 - 100bp DNA ladder (Right)
Lane 2 - *V. harveyi* MTCC strain 3438
Lane - 3to 12 *V. harveyi* isolates

This PCR technique would reduce the risk of luminous disease caused by *V. harveyi* and improve chances of successful harvest. To summarize, this PCR can assist in the confirmation of identity of isolates suspected to be *V. harveyi* much more rapidly and economically than when using phenotypic keys alone. Moreover, the PCR produces less ambiguous results because of the phenotypic diversity of this species. The PCR carried out for all the isolates have shown positive results with no-spurious banding or excessive primer polymerization. The time factor alone indicates that affected animals can be diagnosed in a fraction of the time spent previously with biochemical and morphological testing.

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