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Rapid and specific detection of luminous and non-luminous *Vibrio harveyi* isolates by PCR amplification

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***Vibrio harveyi* is the major causal organism of luminous vibriosis, which causes potential devastation to diverse ranges of marine invertebrates over a wide geographical area. These microorganisms, however, are extremely difficult to identify because they are phenotypically diverse. Biochemical identification techniques involve many tests which may be time-consuming and expensive. The development and sustainability of shrimp aquaculture industry requires a simple, fast and reliable technique for species-specific identification of *V. harveyi* in order to control it effectively. The present communication describes a simple, cost-effective and rapid PCR-based and species-specific detection technique to facilitate early detection and identification of luminous and non-luminous *V. harveyi* isolates. Many of these isolates are also resistant to multiple antibiotics such as ampicillin, chloramphenicol, nalidixic acid, rifampin, polymyxin-B, trimethoprim and penicillin.**

Keywords: Luminous *V. harveyi*, PCR amplification, shrimps, *Vibrio harveyi*.

VIBRIO harveyi, a marine bacterium, is not only ubiquitous in the marine environment but is also considered one of the main bacterial species which is part of normal microflora of healthy shrimps[1]. However, over the past few decades, bacterial strains of this species have been recognized as significant pathogenic agents and a cause of high rates of shrimp mortality in the shrimp culture industry worldwide[2,3]. In various parts across the globe, *V. harveyi* drastically affects the production of *Litopenaeus vannamei*[4], the most extensively cultured penaeid in all the zones. Vibriosis, especially luminous disease has caused serious loss in prawn hatcheries. *V. harveyi* was reported as the causative bacterium of vibriosis in pearl oyster (*Pinctada maxima*), black tiger prawn (*Penaeus monodon*) and kuruma prawn (*Penaeus japonicus*)[5,6]. Larval prawns are particularly susceptible to *V. harveyi*, succumbing to what has been termed as luminescent bacterial disease[6]. This disease has been identified as a major problem in the Philippines, Japan, Southeast Asia and European countries, causing severe losses of juvenile prawns in several hatcheries[7].

Among the common technologies used for diagnosis and detection of *V. harveyi* in shrimp farms are biochemical tests[8,9]. Although these phenotype-based identifications of marine bacteria are useful, they are time-consuming and can generate false-positive results[1]. The development and sustainability of shrimp aquaculture industry urgently requires a simple reliable and fast method for species-specific identification of *V. harveyi* for its adequate control. Likewise several highly powerful molecular techniques, e.g. ELISA, amplified fragment length polymorphism (AFLP) and repetitive extragenic palindromic elements polymerase chain reaction (REP–PCR), FAFLP, and IGSPCR have become readily available for the identification of bacteria, including *Vibrios*[10–14]. Keeping in view these important facts, we have developed a simple, cost-effective reliable and fast PCR-based technique to identify *V. harveyi* isolates. This genomic approach used for the identification and typing of *Vibrio* strains is useful for taxonomic studies, including identification up to the subspecies level. The genus *Vibrio*[15,16] contains a large number of closely related bacterial species with 16 S rRNAs differing in nucleotide sequence from less than 1 up to 6%. Our results have clearly demonstrated the rapid detection of marine luminous and non-luminous *V. harveyi* isolates for molecular epidemiology purpose. This approach may also be applied to the detection of other marine *Vibrio* species involved in aquaculture diseases. These *V. harveyi* isolates were also screened for antibiotic sensitivities[17]. Interestingly, several isolates were found to be resistant to ampicillin, chloramphenicol, nalidixic acid, rifampin, polymyxin-B, trimethoprim, and penicillin; few isolates were sensitive to tetracycline, streptomycin and novobiocin.

Environmental isolates of luminous and non-luminous *V. harveyi* screened were obtained from sea water samples collected from various sampling sites along the west coast of Goa, i.e. Goa Shipyard Limited, Western India Shipping Limited and beaches such as Majorda, Benaulim, Miramar, Donapaula, Anjuna, Tirakol, Colva and Kakra

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(at 15°N lat. and 73°E long.; Figure 1). To enumerate the *Vibrio* sp., 10 ml of sea water was filtered through Whatman no. 1 filter paper and bacteria were subsequently collected on 0.4 µm pore size (Millipore) membrane filters. Bacteria in the membrane were resuspended by vortexing in 2 ml of alkaline peptone water. Thereafter, 100 µl of the bacterial suspension was streaked on thiosulphate citrate bile sucrose agar (selective agar for *Vibrio* sp.) plates containing 2% NaCl. These plates were incubated overnight for 16–18 h at 30°C and the emerging colonies were counted (Figure 2). All the bacterial strains (luminous and non-luminous *V. harveyi* isolates) used in this study have been previously confirmed using the method of Alsina and Blanch's set of biochemical keys for identification of environmental *Vibrio* isolates[9] (data not shown).

V. harveyi strains were cultured in tryptic soy broth supplemented with 2% NaCl at 30°C with continuous shaking until the stationary phase of growth was reached. DNAs were extracted and purified. Bacterial cultures (50 ml) were harvested by centrifugation at 10,000 *g* for 10 min. The resultant pellets were lysed with a 1% sodium dodecyl sulphate–1 mg/ml, proteinase K solution, and the bacterial nucleic acids were extracted by a phenol–chloroform–isoamyl alcohol (25 : 24 : 1, vol/vol/vol) mixture as described by Sambrook *et al*[18]. Extracted DNAs were resuspended in 1X Tris-EDTA buffer and stored at –20°C until required. The DNA sample was subjected to PCR amplification.

PCR was carried out using species-specific primers, VH1 F′ (5′ ACC GAG TTA TCT GAA CCT TC 3′) and VH2 R′ (5′ GCA GCT ATT AAC TAT ACT ACT 3′)[19], which have the ability to cause specific amplification of a 413 bp fragment of the 16S rRNA sequences from a number of *V. harveyi* isolates. The reaction mixture contained 5 µl of 10X polymerase buffer, 3 mM magnesium chloride, 200 µM each dNTP, 1 U per 50 µl *Taq* polymerase, 10 pmol each primer and 50–250 ng template genomic DNA per 50 µl. The thermal cycler was programmed to perform 35 cycles consisting of initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min followed by a final extension step of 5 min at 72°C for 2 min. The same primers were used to set up a positive control with *Escherichia coli* DNA and a negative control with sterile double-distilled water. On gel electrophoresis of 10 µl of PCR product through 0.8% agarose gel and staining with ethidium bromide, amplification was visualized, in order to confirm the size of the PCR product. The 50 and 100 bp DNA ladders were used as standard markers, and the agarose gel picture with PCR product was recorded by gel documentation system (Figure 3).

Stock cultures of *V. harveyi* strains were grown on tryptic soy agar supplemented with 2% NaCl at 30°C for 24 h. The bacteria were suspended in sterile PBS buffer (0.9% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 10% glycerol, pH 7.2) and diluted to a turbidity equivalent to optical density of 0.1 at 600 nm. The bacterial suspension (0.1 ml) was spread onto Mueller–Hinton agar and then antibiotic discs were dispensed in the centre of the plate. The plates were incubated at 30°C for 18 h and the inhibition zones of the bacteria were determined (unpublished data). Minimal inhibitory concentrations for ten antibiotics were determined by the agar dilution method on Mueller–Hinton broth[20]. Each antibiotic was initially dissolved in appropriate solvent. Based on these experiments, percentage of resistant and sensitive *V. harveyi* isolates was determined (Table 1).

The species-specific PCR primers VH-1 and VH-2 corresponded to variable regions of *Escherichia coli* 16S sequence (GenBank accession J01859) at bases 59–87 and 453–473. It was consequently found that these primers corresponded to *Vibrios*, as described by Dorsch *et al.*[15]. The PCR amplified product of VH-1 and VH-2 is 413 bp from all the isolates of non-luminous and luminous *V.*

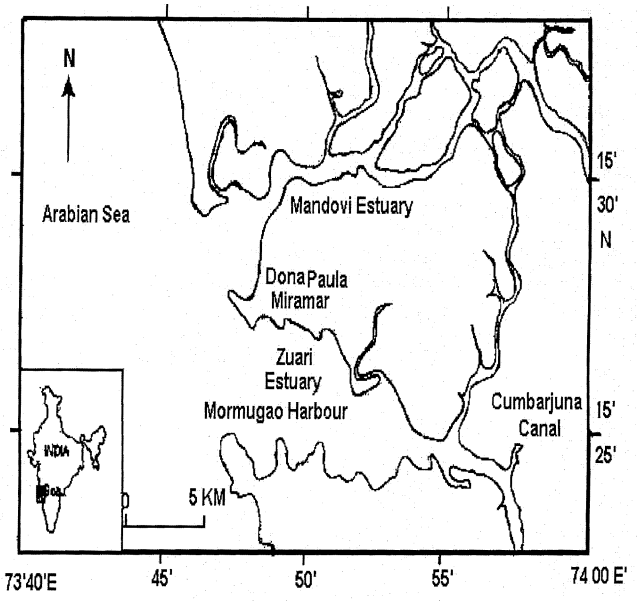

Figure 1. Map showing sampling sites for collection of luminous and non-luminous *Vibrio harveyi* isolates.

Table 1. Antibiotic sensitivity of *Vibrio harveyi* isolates (luminous and non-luminous) from different sites in the coastal regions of Goa

Antibiotic (µg/ml)	No. of isolates tested	Per cent resistant isolates
Ampicillin (75)	34	88.23
Chloramphenicol (50)	34	70.58
Tetracycline (25)	22	36.36
Streptomycin (25)	34	29.41
Nalidixic acid (50)	22	72.72
Rifampin (25)	22	81.82
Novobiocin (50)	22	54.52
Polymyxin-B (75)	22	63.63
Trimethoprim (75)	22	85.29
Penicillin (75)	22	77.27

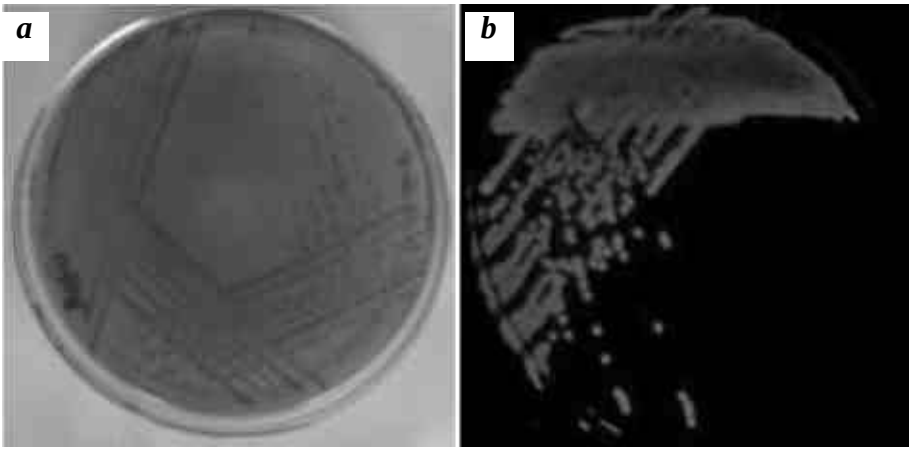

Figure 2. *V. harveyi* colony on TCBS agar (***a***) and glycerol-based marine agar (***b***) plates.

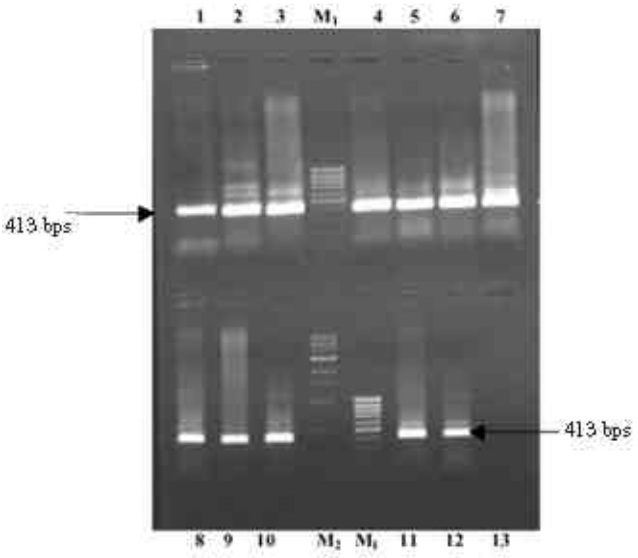

Figure 3. Agarose gel electrophoresis of 16S ribosomal DNA (PCR products) from 12 representative bacterial isolates (*V. harveyi*). Lane M_2, 50 bp and Lane M_1, 100 bp molecular weight markers; lanes 1–12, Isolates (1–7, isolates of luminous *V. harveyi* and 8–12, isolates of non-luminous *V. harveyi*); lane 13, Negative control, *Escherichia coli*.

harveyi. This PCR method has been found to reduce the time and resource required for confirmation of identity of *V. harveyi.* The identification of *Vibrios* isolated from the aquaculture environment has been imprecise and labour-intensive, requiring many biochemical and physiological tests[21], or dichotomous keys that takes weeks to perform[9]. With our PCR-based technique, an isolate suspected to be luminous or non-luminous *V. harveyi* could be confirmed in less than 24 h. The positive-reacting cultures would require growth characteristics, VP test, and use of mannose, L-leucine, acetate or propionate as a sole carbon source, to confirm the identity of *V. harveyi* isolates. However, this PCR-based taxonomic identification of *V. harveyi* could easily initiate antibacterial therapy and thus prevent the rapid spread of specific *V. harveyi* in the estuarine and freshwater aquaculture.

The antibiotic sensitivity study revealed that *V. harveyi* developed resistance to many of the antibacterial agents tested. Most of the Gram-negative bacteria acquire resistance to antibacterial agents by changing the permeability of outer membrane porin channels, consequently leading to reduced drug influx into the bacterial cell and antibiotic resistance gene determinants present on the plasmids, transposons and integrons[22]. A large number of *V. harveyi* strains were found to be resistant to ampicillin (88.23%), chloramphenicol (70.58%), nalidixic acid (72.72%), rifampin (81.82%), polymyxin-B (63.63%), trimethoprim (85.27%), and penicillin (77.27%; Table 1). Occurrence of these antibiotic-resistant *V. harveyi* isolates is mainly due to the continuous usage of several broad spectrum antibiotics in the aquaculture sites. From the above results, it can be inferred that the virulence of the luminous and non-luminous *V. harveyi* strains increased slightly due to increased resistance to various antibacterial agents, including antibiotics.

This PCR-based detection technique facilitates the identification of bacterial isolates suspected to be *V. harveyi*, much more rapidly and economically than any other conventional techniques used for detection. This technique is also cost-effective, simple and fast, therefore *V. harveyi* can be controlled effectively and efficiently.

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Detection of genetic variability among chrysanthemum radiomutants using RAPD markers

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Eleven radiomutants from two chrysanthemum cultivars Ajay and Thai Chen Queen were characterized by RAPD to understand the extent of diversity and relatedness. Out of 40 random primers screened, 21 gave reproducible polymorphic bands. PCR product of radiomutant genome revealed a total of 156 bands, out of which 118 were found to be polymorphic. Cluster analysis of the radiomutants indicated that they fell into three major groups. Yellow and Bright Orange mutants derived from cv. Thai Chen Queen have been placed in a separate group, indicating their high genetic diversity from the rest of the mutants and parents. The study revealed that RAPD molecular markers can be used to assess polymorphism among the radiomutants and can be a useful tool to supplement the distinctness, uniformity and stability analysis for plant variety protection in future.

Keywords: Chrysanthemum, genetic variability, polymorphism, radiomutants, RAPD markers.

CHRYSANTHEMUM is the second largest cut-flower after rose among the ornamental plants traded in the global flower market. It is cultivated both as a cut-flower and as a potted plant (pot mums). The commonly grown chrysanthemums are hexaploid complex with average number of 54 chromosomes[1]. It is propagated vegetatively as it has a strong sporophytic self-incompatibility system as shown by all members of Asteraceae family[2].

Mutation breeding by radiation, an agricultural application of nuclear technology has been widely utilized to upgrade the well-adapted plant varieties by one or two major traits and also develop new varieties with improved agricultural characteristics. Although most cultivated chrysanthemum cultivars are polyploids with high genetic heterogeneity, mutants with altered flower colour, shape, floret size and shape are often recovered. Altered flower colours with chimeric tissue can be easily induced by radiation and can be isolated using *in vitro* tools. Identification and characterization of cultivars is extremely important in horticultural crops in order to protect the plant breeders' rights. Earlier, new varieties were identified based on horticultural and physiological parameters. New cultivars of chrysanthemum are developed from a single progenitor either spontaneously (sports) or by radiation-induced mutation[3]. Since the effect of mutation in ornamentals is clearly visible, selection for changed flower colour, shape, and size is possible in the M1 generation itself because most of the ornamental crops are vegetatively propagated. Novelty visible in any form is of high value in ornamental crops and hence mutation breeding played a key role in the improvement of ornamental crops in general and chrysanthemum in particular.

Williams *et al.*[4] developed DNA fingerprinting using RAPD markers during 1990. Since then, chrysanthemum cultivars and other closely related family members of Asteraceae have been characterized based on RAPD[1,5,6] and DAF analysis in other parts of the world. Genetic variation between two genetically diverse tissues and three chimeral cell types in leaf has also been demonstrated earlier using the RAPD technique[1].

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