**RESEARCH ARTICLE**

# Evaluation of RAPD-PCR and protein profile analysis to differentiate *Vibrio harveyi* strains prevalent along the southwest coast of India

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## Abstract

Sixty five isolates of *Vibrio harveyi* were subjected to random amplified polymorphic DNA (RAPD)-PCR analysis and protein profiling to investigate the genetic variability among *V. harveyi* prevalent along the coast and also assess the discriminating ability of these two molecular methods. A total of 10 RAPD primers were assayed for their specificity in detecting *V. harveyi*, of which only two primers: PM3 and CRA25 were highly reproducible and found suitable for use in RAPD-PCR. The genetic diversity among *V. harveyi* isolates assessed by RAPD-PCR using PM3 primer yielded 35 different RAPD patterns which clustered the isolates into 15 groups at 72% similarity level. Similarly, RAPD-PCR with CRA25 clustered the 38 patterns into 10 groups at 74% similarity. The discriminatory index (*D*) value calculated for RAPD fingerprints generated with PM3 and CRA25 were 0.90 and 0.85, respectively. On the other hand, molecular typing of *V. harveyi* using whole cell proteins generated profiles that showed no major difference indicating the technique to be not useful in typing strains of this bacterium. However, a few of the isolates showed the presence of unique band of 28 kDa that needs to be further investigated to understand the role of the protein in disease process if any.

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## Introduction

*Vibrio harveyi* is a Gram-negative bioluminescent marine bacterium, occurring either as a free-living form or in association with intestinal microbiota of marine animals (Makemson and Hermosa 1999). While some strains are highly pathogenic to aquatic fauna; especially invertebrates, others are considered to be opportunistic pathogens (Oakey *et al*. 2003). As a pathogen, *V. harveyi* manifests itself as luminous vibriosis in hatchery reared and commercially farmed penaeid shrimps resulting in severe economic losses to shrimp industry in Asia (Lavilla-Pitogo *et al*. 1990; Karunasagar *et al*. 1994; Conejero and Hedreyda 2003). As with other bacterial species, there is a need for specific typing of *V. harveyi* for epidemiological purposes to enable the tracking of strains dispersed in the environment and to understand the spread of the bacterium.

Several polymerase chain reaction (PCR)-based DNA fingerprinting methods exist for molecular typing of bacterial species. Randomly amplified polymorphic DNA (RAPD) is a PCR-based technique, using arbitrary primers to detect changes in the DNA sequence at sites in the genome. This approach is used for molecular epidemiological typing as it is relatively fast and easy (Williams *et al*. 1990). The polymorphism within the set of DNA fragments generated has been used in discriminating microorganisms both at the interspecies and intraspecies level (Welsh and McClelland 1993). In several *Vibrio* spp. this technique has been used in diversity studies (Shangkuan *et al*. 1997; Somarny *et al*. 2002), and in *V. harveyi* it has been used in differentiating pathogenic and nonpathogenic strains (Somarny *et al*. 2002; Hernandez and Olmos 2004; Alavandi *et al*. 2006). Another molecular technique that has proved to be useful in typing

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bacterial strains is sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell bacterial proteins, wherein differences seen in protein bands have been successfully used to group bacteria (Krech *et al*. 1988; Huey and Hall 1989). *V. harveyi* strains are reported to express different proteins in different circumstances (Pizzutto and Hirst 1995). Against this background, we aimed to study the genetic diversity of *V. harveyi* prevalent along the southwest coast of India using RAPD-PCR and protein profile typing methods which could help understand the epidemiology and ecology, tracking outbreaks and spread of this organism.

## Materials and methods

### *Bacterial cultures and DNA isolation*

*V. harveyi* stock cultures ($n = 65$) maintained in tryptic soy broth with 30% glycerol at −80°C were revived and plated onto luminescent agar plates (West and Colwell 1984). A single luminescent colony of each culture was inoculated in tryptic soy broth with 1% NaCl and grown overnight at 37°C. Genomic DNA was extracted and purified according to Ausubel (1995). Briefly, the bacterial cell pellet was suspended in 567 $\mu$L of 1× TE buffer (10 mM Tris-Cl; 1 mM EDTA; pH 8.0), 30 $\mu$L of 10% Sodium dodecyl sulphate and 3 $\mu$L of proteinase K (20 mg/ml) and incubated at 45°C for 1 h. Hundred $\mu$L of 5 M NaCl and 80 $\mu$L of CTAB/NaCl solution was added and the mixture incubated for a further 10 min at 65°C. The solution was centrifuged at 10000 rpm for 10 min with an equal volume of chloroform/isoamyl alcohol. The aqueous phase was transferred to a fresh tube and 0.6 volume of isopropanol added to precipitate DNA which was pelleted by centrifugation and washed with 70% ethanol. The DNA pellet was resuspended in 1× Tris-EDTA buffer (pH 8.0) and checked for its concentration and purity using a spectrophotometer (Shimadzu 1601, Kyoto, Japan) and stored at −20°C until further use. *V. harveyi* strains used in this study are listed in table 1.

### *PCR amplification of the hemolysin gene*

Molecular identity of the cultures was tested based on PCR targeting the hemolysin (*vhh*) gene (Conejero and Hedreyda 2004). Crude bacterial lysates of all cultures were prepared

**Table 1.** List showing details of *V. harveyi* isolates.

| Sampling site | Isolated from | Isolate nos. | RAPD cluster |
|---|---|---|---|
| Goa ($n = 2$) | Adult shrimp | V32 | A3 |
| | Pond water | V33 | A3 |
| Karwar ($n = 22$) | Hatchery water | Vh2, Vh7, Vh9, Vh10, Vh11 and Vh12 | A10, A12 and A13 |
| | Hatchery shrimp larvae | Vh16, Vh18, Vh19, Vh20, Vh21, Vh22 and Vh23 | A10 and A11 |
| | Shrimp pond water | Vh32, Vh33 Vh34, Vh35, Vh36 and Vh37 | A5 |
| | Shrimp pond sediment | Vh24, Vh26 and Vh28 | A5 |
| Kundapur ($n = 30$) | | | |
| Farm 1 | Pond water | V6, V7, V8 and V9 | A14 |
| | Adult shrimp | V14 | A15 |
| Farm 2 | Pond water | V12 and V13 | A15 |
| | Adult shrimp | V16 | A15 |
| Farm 3 | Pond water | V21 , V22, V28 and V34 | A2, A3 and A7 |
| | Adult shrimp | V17 | A15 |
| Farm 4 | Pond water | V45 | A6 |
| | Adult shrimp | V29, V30 and V31 | A3 |
| Farm 5 | Pond water | V35, V40 and V44 | A1, A2 and A6 |
| | Adult shrimp | V36, V38, V41 and V42 | A1 |
| | Wild shrimps | V11 and V18 | A15 |
| | Pond shrimp larvae | V23, V25 , V26 and V27 | A3 and A7 |
| Sasthan ($n = 4$) | Estuary water | V1 | A2 |
| | Pond water | V10 and V39 | A4 and A14 |
| | Diseased shrimp | V37 | A4 |
| Udupi ($n = 1$) | Estuary water | V43 | A4 |
| Hejamadi ($n = 4$) | Moribund shrimp | V2 and V3 | A2 |
| | Pond water | V4 and V5 | A8 and A9 |
| Kannur ($n = 2$) | Hatchery shrimp larvae | V19 | A7 |
| | Hatchery water | V20 | A7 |

as described by Dileep *et al*. (2003). PCR primers used for targeting a 235-bp internal fragment of the gene were as follows; forward: 5-CTTCACGCTTGATGGCTACTG-3, and reverse: 5-GTCACCCAATGCTACGACCT-3. PCR was carried out in a 30-$\mu$L reaction mixture consisting of 3 $\mu$L of 10× PCR buffer (100 mM Tris-HCl pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 200 $\mu$M of each dNTPs, 25 pmol of each primer, 0.75 U of *Taq* polymerase (Bangalore Genei, Bangalore, India) and 2 $\mu$L of template DNA. PCR was carried out in a thermocycler (MJ Research, Watertown, USA) with the reaction cycles consisting of an initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension of 72°C for 5 min. The PCR products were resolved on 2% agarose gels containing 0.5 $\mu$g/mL ethidium bromide and documented using a gel documentation system (Herolab, Wiesloch, Germany). Reference strains of *V. parahaemolyticus* (ATCC154, USA) and *V. harveyi* (VH027-LMG07890, Belgium) were used as negative and positive control, respectively.

***RAPD-PCR assay***

Ten randomly designed 10-mer oligonucleotide primers (table 2) were initially used for screening DNA samples to obtain reproducible RAPD fingerprints. RAPD-PCR was run thrice to evaluate and check for the repeatability of the fingerprints generated. Out of the six PM primers (Tassanakajon *et al*. 1997) and four CRA primers (Neilan 1995) tested, only two primers viz. PM3 and CRA25 provided consistent well resolved and reproducible band patterns and were therefore selected for further analysis. The PCR reaction mixture of 50 $\mu$L consisted of 5 $\mu$L of 10× PCR buffer (100 mM Tris-HCl pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 250 $\mu$M of each deoxynucleotide triphosphates, 50 pmol of primer, 1.5 U of *Taq* polymerase (Bangalore Genei, Bangalore, India). Standardized PCR thermocycling conditions for the RAPD included initial denaturation at 95°C for 5 min and final delay at 72°C for 5 min followed by 35 cycles, 95°C for 45 s, 36°C for 45 s, 72°C for 1 min and 95°C for 30 s, 45°C for 30 s, 72°C for 1 min for PM and CRA series of primers, respectively. The amplification was carried out in a thermocycler (MJ Research, Watertown, USA) and the products resolved in 1.6% agarose gel, stained with ethidium bromide and the results photograph captured by Gel Documentation system (Herolab, Wiesloch, Germany).

***RAPD data analysis***

All fragments generated by RAPD-PCR using the two primers were considered and analysed separately for determining the RAPD types. The size of each band was determined using Kodak Digital Science 1D (KSD1D 2.0, Rochester, USA) software and the presence (1) or absence (0) of a particular band was recorded to generate a binary table. The data table was exported into the Popgen32 software (Yeh and Boyle 1997) for analysis. The normalized RAPD pattern was further analysed using Gel compare II version 2.5 (Applied Maths, St Martens-Latem, Belgium). The levels of similarity in the RAPD fingerprints were calculated using Pearson's correlation coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) method. The relationships between the pattern profiles are displayed as dendrograms and expressed as percentage similarity. The numerical discriminatory index (*D*) which is a measure of the discriminatory ability of the typing methods was calculated according to Hunter and Gaston (1988).

**Table 2.** Primers sequences used in RAPD-PCR.

| Primer | Sequence (5′–3′) | Reference |
|---|---|---|
| PM1 | GCCCCTGGAG | Tassanakajon *et al*. (1997) |
| PM2 | AACGGGCAGG | Tassanakajon *et al*. (1997) |
| PM3 | GGCTGCGGTA | Tassanakajon *et al*. (1997) |
| PM4 | GCGGAGGTCC | Tassanakajon *et al*. (1997) |
| PM5 | CGACGCCCTG | Tassanakajon *et al*. (1997) |
| PM6 | GCGTCGAGGG | Tassanakajon *et al*. (1997) |
| CRA22 | CCGCAGCCAA | Neilan (1995) |
| CRA23 | GCGATCCCCA | Neilan (1995) |
| CRA25 | AACGCGCAAC | Neilan (1995) |
| CRA26 | GTGGATGCGA | Neilan (1995) |

***Protein profiling***

All 65 isolates of *V. harveyi* were tested for their protein profiles. Each bacterial culture grown overnight in tryptic soy broth was pelleted by centrifugation at 10000 rpm for 10 min. Bacterial pellets were resuspended in 50 $\mu$L of 2× sample buffer (0.5 % sodium dodecyl sulphate, 1.25 % 2-b-mercaptoethanol, 0.03 % bromophenol blue, 2.5 % glycerol in 15 mM Tris-C1 at pH 6.8) and incubated in a dry bath at 98°C for 15 min. Approximately 40 $\mu$L of the protein sample was taken and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) containing 5% stacking and 10% of resolving gels and separated based on Laemeli discontinuous buffer system (Harlow and Lane 1988). After electrophoresis on a vertical slab unit under a constant voltage of 150 V for 3 h, the gels were stained with coomassie brilliant blue R-250 (HiMedia, Mumbai, India). A large protein marker calibration kit (Bangalore Genei, Bangalore, India) was used to estimate the molecular weight of protein bands.

## Results

***PCR for vhh gene***

All 65 isolates amplified the expected 253 bp of the *vhh* gene (figure 1) indicating the presence of a hemolysin gene, thereby, further confirming the identification of *V. harveyi* used in this study.

***RAPD analysis***

In this study, RAPD-PCR fingerprints were generated for 65 isolates of *V. harveyi* using the primers CRA25 and PM3.

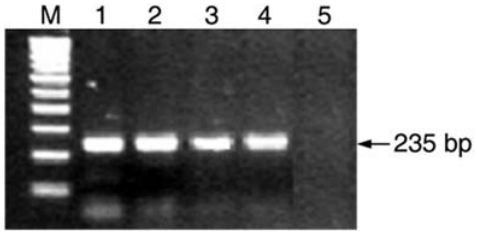


**Figure 1.** PCR amplification of the hemolysin gene. Lane M, 100-bp DNA ladder (GeNei$^{TM}$, Bangalore, India); lane 1, positive control (VH027-LMG07890); lane 2, Vh2 isolate; lane 3, Vh23 isolate; lane 4, Vh7 isolate; lane 5, *Vibrio parahaemolyticus* (ATCC154).

Primer PM3 amplified 2–9 fragments with band sizes ranging from 0.25 to 2.0 kb, whereas CRA25 yielded 2-11 bands, with amplicon sizes ranging from 0.2 to 4.0 kb. Cluster analysis of primers CRA25 and PM3 generated RAPD profiles separated the isolates at an average similarity values of 74% and 72%, respectively (data not shown). Primer CRA25 yielded 38 different patterns which clustered the isolates into 10 RAPD types; while primer PM3 yielded 35 patterns that clustered into 15 RAPD types. A dendrogram based on the combined similarity matrix generated with the two RAPD primers is presented in figure 2. The combined dendrogram at an average similarity value of 73% grouped all strains into 15 (A1–A15) clusters showing high diversity in profiles. Among the strains, nine (13.85%) belonged to A5, seven (10.77%) each to A3, A7 and A15, six (9.23%) to A10, five (7.69%) each to A1, A2, A14, four (6.16%) to A13, three (4.62%) to A4, two (3.01%) each to A6, A11 and the remaining three showed individual distinct patterns designated as A8, A9 and A12 (figure 2). Besides, the RAPD profiles also enabled a few of the isolates to be discriminated based on their geographical source of isolation. As seen in figure 1, the clusters A1, A6 and A15 were derived from Kundapur isolates, while A5, A10, A11 and A13 were from Karwar. The remaining clusters consisted of mixed geographical strains. The grouping observed among the geographical isolates in clusters A10, A11, A13 and A15 was common to that observed in profiles generated by PM3 and CRA25 primers. Eight of the nine isolates in A5 and two isolates in A6 cluster presented identical grouping with PM3 but generated different profiles with CRA25. Similarly, A15 presented as a single cluster with CRA25, but formed two subgroups with PM3 at 68% similarity. The discriminatory index (*D*) calculated for CRA25, PM3 and combined dendrograms were 0.85, 0.90 and 0.93, respectively. It was also observed that with the CRA25 primer 96.9% isolates amplified a common fragment of 800 bp. Similarly, with PM-3 primer two bands of sizes 700 and 850 bp were common in 89.2% and 70.8% of the isolates, respectively.

***Protein profiling***

On 10% SDS-PAGE electrophoresis, the 65 isolates of *V. harveyi* analysis yielded approximately 13–18 clear and distinct polypeptide bands with molecular weights ranging from 10 to 100 kDa. The protein banding patterns were observed to be identical for all the isolates tested. However, in three of the isolates (V1, Vh24 and V37), an additional band of 28 kDa was detected (figure 3).

## Discussion

Molecular characterization techniques are now widely used both for ecological and epidemiological analyses of a wide range of bacterial species. In this study, we have evaluated two molecular methods, RAPD-PCR and bacterial whole cell protein profiling to differentiate polymorphic *V. harveyi* prevalent along southwest coast of India. RAPD-PCR was exploited using two primers: CRA25 and PM3 which gave reproducible and well distinguishable amplicons. The RAPD profiles obtained with primers CRA25 and PM3 individually, showed diversity among *V. harveyi* isolates used in this study. Cluster analysis based on combined similarity matrix grouped all strains into 15 clusters, indicating a genetically heterogeneous group of *V. harveyi* to be prevalent along the coast. Our observations are in agreement with previous studies in reporting the presence of a large number of heterogenic genotypes within *V. harveyi* (Pujalte *et al*. 2003; Hernandez and Olmos 2004; Alavandi *et al*. 2006). Additionally, the ability of RAPD-PCR to cluster *V. harveyi* strains suggests that this technique could be used in differentiating *V. harveyi* strains. From this study, it was observed that among the CRA25 generated amplicons, one amplicon of size 800 bp was shared by almost all *V. harveyi* isolates studied. Similarly with PM-3, two bands of sizes 700 bp and 850 bp were common to nearly all strains. A similar observation was also made by Pujalte *et al*. (2003), who reported that most of the *V. harveyi* isolates in their study amplified a common band of 800 bp when subjected to RAPD-PCR using Primer M13. The sharing of common bands indicates the presence of a highly conserved genomic region in diverse *V. harveyi* strains in this region. This assumes significance as amplification of common fragments by RAPD-PCR with a particular primer has been shown to be useful in genetic amplifications and hybridization assays for diagnostic purpose (Dalla *et al*. 2002). Further, these highly conserved fragments could be ideal for identifying strains that are atypical or which may be difficult to identify by phenotypic tests. Hunter and Gaston (1988) suggested that an index value greater than 0.90 is desirable if the discrimination levels for RAPD typing are to be interpreted with confidence. The discriminatory values obtained for the two primers highlight that RAPD-PCR with primer PM3 (0.90) as having higher potential to detect genetic variations and better suited for differentiating strains of *V. harveyi*. However, a combined analysis of the two RAPD profiles (CRA25 and PM3) resulted in a higher discriminatory value of 0.93 and hence we suggest that for establishing a higher discriminatory relationship among isolates a combination of these two fingerprints should be used.

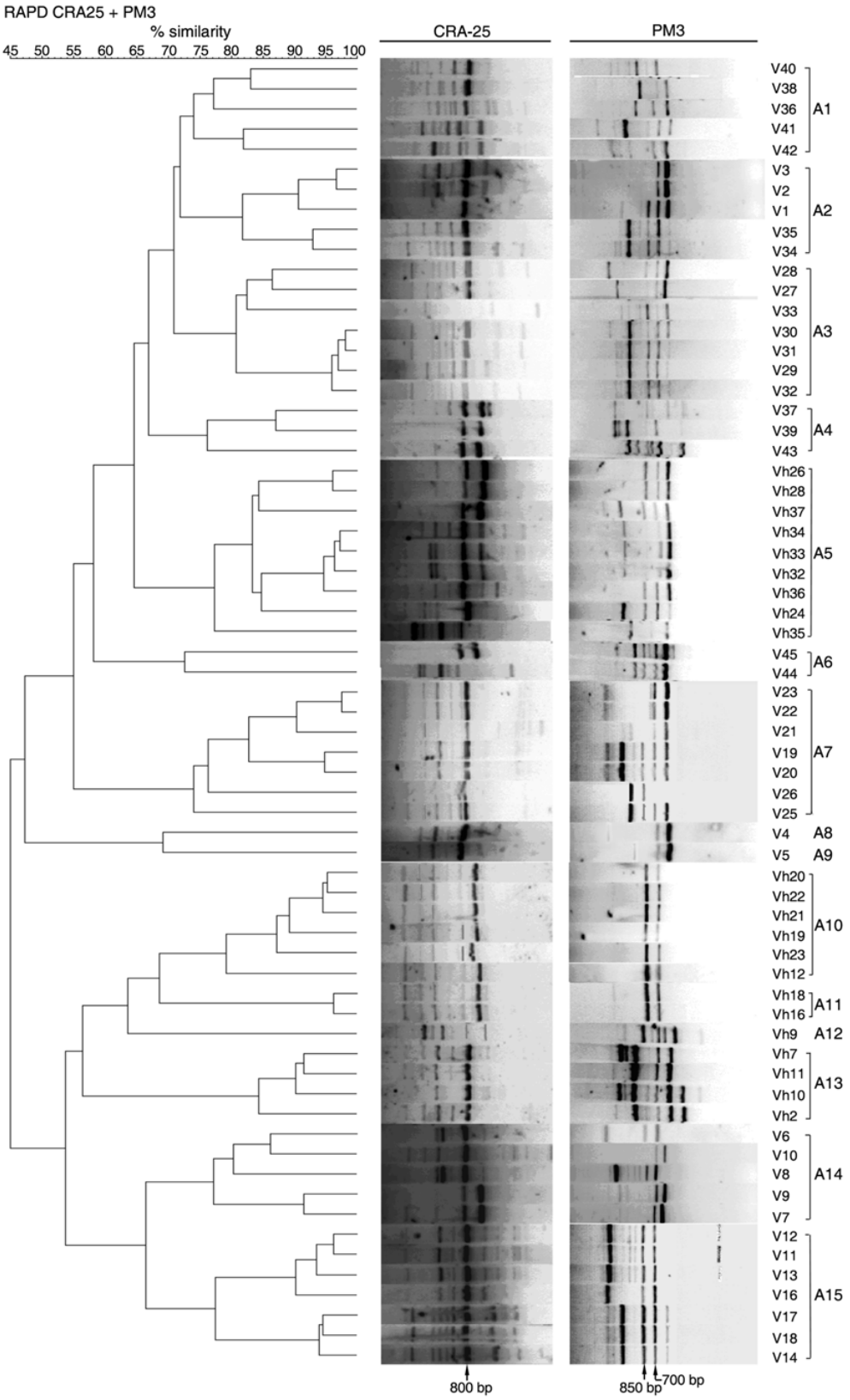


**Figure 2.** Combined dendrogram for *V. harveyi* isolates generated by combining the RAPD-PCR profiles obtained by CRA25 and PM3 primers. A1–A15 represent the different RAPD types resolved at 73% similarity. '*D*' value calculated was 0.925. Arrows indicate the conserved bands observed in the fingerprints.

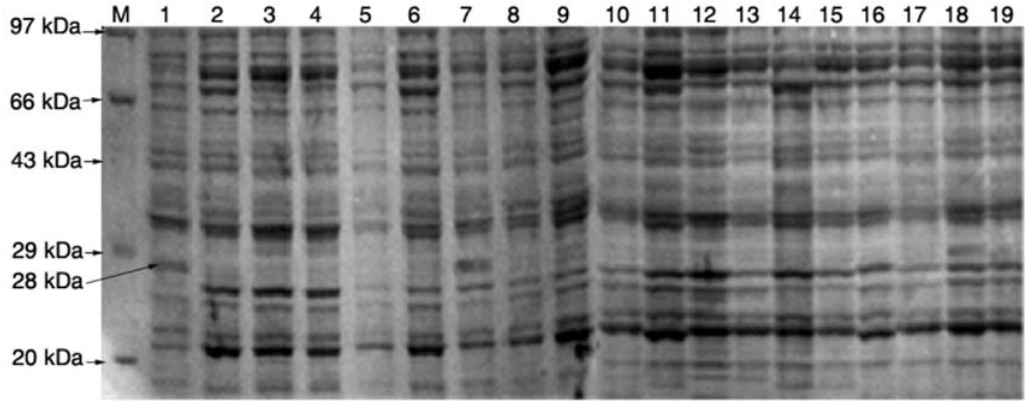


**Figure 3.** Representative protein profiles of *V. harveyi* isolates. M, protein marker; lanes 1–19, *V. harveyi* isolates; lane 1, estuary water isolate (V1); lane 7, diseased shrimp isolate (V37); lane 18, pond sediment isolate (Vh24).

RAPD typing method allows the tracing of particular strains and its prevalence in different hosts. Although several of our samples tested from the same location shared the same RAPD type (table 1), no correlation was obtained between a given RAPD type and the geographical location or the source of the isolates. Further, *V. harveyi* isolated from shrimps in this study were all from healthy animals except for one, V37 (Sasthan), which was from a diseased shrimp. Interestingly, *V. harveyi* from the pond water (V39) where the shrimp was sampled belonged to the same RAPD type which probably suggests that there could have been a horizontal transmission of the bacterium from water to shrimp. Further studies are required to clearly establish an association between a particular RAPD type to virulence and disease, which could have important implication in establishing the origin of a pathogenic strain. This could be particularly useful in molecular epidemiology studies for tracing the route of infection for implementing suitable control measures for this bacterium.

A study by Pizzutto and Hirst (1995) showed that *V. harveyi* isolates could be grouped based on their whole-cell protein profiles generated on SDS-PAGE. However, in this study the protein profiles failed to differentiate *V. harveyi* isolates into different groups. Except for slight variations in the number of bands generated, the overall profiles for all isolates tested remained the same. However, the expression of the 28-kDa polypeptide band in three of the isolates i.e. V1, V37, and Vh24 requires further investigation. Further, V1 (estuary water) and V37 (diseased shrimp) are isolates from the same geographical area (Sasthan) (table 1) and we do not know whether the expression of this additional band has any role in the pathogenicity of this organism. Results of protein profiling suggest that the technique cannot be used in typing *V. harveyi* strains in this region. However, it could be useful in identifying new proteins which may be helping the organisms in their adaptation to hosts and/or virulence.

In this study, although RAPD-PCR and SDS-PAGE profiles were reproducible and generated several bands, the banding patterns observed with protein profiling were almost similar and not discriminatory as observed with DNA fingerprinting. Thus, it can be concluded that RAPD-PCR which is a rapid and simple tool could be used in typing and differentiating a large number of *V. harveyi* strains, which could be useful in studying the epidemiology and distribution for implementing appropriate measures for controlling disease caused by the bacterium.

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