Characterization of multidrug resistance in *Vibrio* species isolated from marine invertebrates from Andaman Sea

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Abstract

This study describes the abundance of multidrug-resistant *Vibrios* associated with marine invertebrate hosts from the Andaman Sea, India. Thirty-eight *Vibrio* strains were isolated from surface mucus layers of coral *Porites*, *Goniastrea*, *Pocillopora*, *Fungia*, and eggs of spiny lobster (*Panulirus penicillatus*). Phenotypically, the majority of strains exhibited growth at a wide range of temperatures, salt tolerance, and diverse nutritional requirements. All the strains had more than 97% 16S rRNA sequence similarity with type species of the genus *Vibrio* where *Vibrio fortis*, and *Vibrio alginolyticus* were predominant. Multilocus Sequence Analysis (MLSA) using eight housekeeping genes namely *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA* distributed the strains into 6 reported clades i.e., *Harveyi*, *Ponticus*, *Nereis*, *Orientalis*, *Splendidus*, and *Mediterranei* where nearly half of the total strains represented the clade *Harveyi*, followed by the clade *Splendidus*. Likewise, the PFGE profile indicated genomic heterogeneity among the strains resulting in their distribution in five major clusters. Resistance to different antimicrobials was tested following the disc diffusion method where all strains were found susceptible to chloramphenicol (30 µg) and resistant to streptomycin (10 µg), vancomycin (30 µg), sulfamethoxazole-trimethoprim (25 µg). Moreover, the resistant phenotype to other antimicrobials confirmed the abundance of multidrug resistance strains in this marine environment.

Keywords Marine invertebrate · *Vibrio* · Multilocus sequence analysis · Genetic diversity · Drug-resistant

Introduction

Vibrios are ubiquitous in the marine environment across the globe (Thompson et al. 2004; González-Escalona et al. 2008). Rapid growth with a short generation time, salt tolerance, diverse nutritional requirement, and biofilm-forming capacities have acquired the adaptive abilities and

physiological flexibilities in the genus *Vibrio* to survive and flourish in the diverse oligotrophic marine environment (Takemura et al. 2014; Zhang et al. 2018). The marine *Vibrios* initially gained attention due to its pathogenic potentials (Stewart et al. 2008), however, later recognized for its ecological values due to its role in nutrient mineralization and contribution to the oceanic biogeochemical processes (Takemura et al. 2014; Zhang et al. 2018). As such, the genus *Vibrio* is considered as the model marine heterotrophic bacterial groups and an indicator of marine health.

Vibrios have been isolated from marine environments either free-living in seawater (Amin et al. 2016), sediment or associated with marine invertebrates like corals, shrimps, fishes (Thompson et al. 2003; Sawabe et al. 2004; Macián et al. 2004; Pruzzo et al. 2005; Yoshizawa et al. 2010; Sheu et al. 2011). These are either pathogenic (Pruzzo et al. 2005; Austin and Zhang 2006) or non-pathogenic (Thompson et al. 2003).

Compared to the global efforts in analysing marine microbial diversity especially abundance, functions, and drug resistance of the genus *Vibrio* (Tall et al. 2013; Mansergh

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and Zehr 2014; Amin et al. 2016; Lee and Raghunath 2018; Deng et al. 2020), the part of Andaman Sea near the Andaman and Nicobar Islands of Indian subcontinent is least studied. This region harbours rich coral reefs that host the most populous marine life forms and is an excellent yet untapped area of microbial investigation (Gibson et al. 2007). Few studies confirmed the presence of several novel species (Deep et al. 2014; Kumari et al. 2014a, b, 2016) of the genus *Vibrio*. However, *Vibrio* diversity concerning its reef and other marine life has not been explored till date. Considering the Spatio-temporal significance of this region, detailed insight on the *Vibrio* abundance associated with marine invertebrates including the reefs and other marine organisms may provide potential ecological information and could make a way for further scientific endeavours.

In view, this report describes the diversity, genetic variability, and multidrug-resistant properties of some *Vibrio* strains isolated from marine invertebrates from the Andaman Sea. Complete taxonomic resolutions for the strains were obtained by a combination of phenetic, phylogenetic, and molecular studies involving Multi-Locus Sequence Analysis (MLSA) and Pulsed-Field Gel Electrophoresis (PFGE). Further, antimicrobial susceptibility of the strains were assessed to understand the abundance and extent of drug resistance phenotype in this pristine marine environment as a function of ecological health.

Material and methods

Sample collection and isolation of bacteria

Five coral species namely *Porites porites*, *Goniastrea retiformis*, *Pocillopora verrucosa*, *Fungia danai*, *Fungia echinata*, and eggs of a female spiny lobster (*Panulirus penicillatus*) were collected from different locations in the Andaman Sea during November 2010 and March 2012 (Table 1). Seawater temperature and pH during the collection at the site were between 27 and 29 °C and 7.6, respectively (Table 1). Coral samples were collected from the depth of about 3.5–5 m and lobster samples were collected from a depth of about 3.0–3.5 m. Mucus samples of ca. 1 cm² coral surface area from individual coral species were taken using sterile cotton swabs (Guppy and Bythell 2006) and transferred into a sterile tube containing 1 ml of filter-sterilized seawater. The bacteria from the cotton swabs were suspended in seawater by vortexing and used as a master mix. An aliquot (100 µl) of the mixed samples was serially diluted using sterile seawater and plated onto Bacto Marine Agar 2216 (MA, Difco, USA) as described earlier (Kumari et al. 2014a; 2014b). Similarly, eggs on pleopods of a female lobster were removed with a sterile scissor, washed with filter-sterilized seawater, and collected in a sterile tube containing 5 ml sterile seawater. Samples were transported to the laboratory and the bacteria were isolated following the methods described

Table 1 Metadata of samples used in this study

Geographical location	Date of sample collection; sea temperature; pH	Strain(s) isolated
Lobster (<i>Panulirus penicillatus</i>)		
Near Henry Lawrence Island (12° 05' 7.3"N, 93° 4' 11.8"E)	20.03.2012; 27 °C; 7.6	LBS1, LBS3, LBS4, LBS6, LBS9, LBS11, LBS12
Coral (<i>Porites porites</i>)		
Near John Lawrance Island (12°02'34.3"N 93°02'19.1"E)	20.03.2012; 27 °C; 7.6	S1-10, S1-11, S1-14, S1-15, S1-17, S1-18, S1-20
Between John Lawrance and Henry Lawrance (12°05'33.6"N 93°03'44.0"E)	20.03.2012; 27 °C; 7.6	S3-31, S3-32
Coral (<i>Goniastrea retiformis</i>)		
Near North Bay (11°42'14.0"N 92°45'05.7"E)	21.03.2012; 28 °C; 7.6	S2-24, S2-25, S2-26
Coral (<i>Pocillopora verrucosa</i>)		
Near North Bay (11°42'14.0"N 92°45'05.7"E)	21.03.2012; 28 °C; 7.6	S6-61
Coral (<i>Fungia danai</i>)		
Between John Lawrance and Havelock (12°01'33.8"N 93°00'36.7"E)	20.03.2012; 27 °C; 7.6	S7-71, S7-72, S7-73, S7-74A, S7-74B, S7-77
Water samples (sea surface)		
Between John Lawrance and Henry Lawrance (12°05'26.9"N 93°03'51.9"E)	20.03.2012; 29 °C; 7.6	W9
Coral (<i>Fungia echinata</i>)		
Between Havelock Island and Elephant Wall (12°00'39.5"N 92°56'43.2"E)	23.11.2010; 27 °C; 7.5	AN11, AN12, AN14, AN38, AN43, AN60, AN61, AN62, AN72, AN81, AN82

earlier (Deep et al. 2014). For the isolation of bacteria from water, aliquots of 100 ml water samples were collected at different locations between John Lawrance and Henry Lawrance Island. The samples were pooled (total 1000 ml) and filtered through a 0.22 µm Millex membrane filter. The membrane was rinsed with 5 ml sterile saline water and the 100 µl of this wash solution was plated onto MA for the isolation of bacteria. All plates were incubated at 28 °C for 4 days. Colonies that appeared on MA plates were picked up, purified by repeated streaking on the same medium. Strains were identified based on colony morphology, Gram staining followed by 16S rRNA sequence analysis. Pure cultures were preserved in 15% glycerol at – 80 °C for the long term and at 4 °C for the short term.

Phenotypic characterization

For phenotypic analysis, strains were grown at 28 °C for 48 h, unless mentioned otherwise. Tests like oxidase, catalase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrolysis of urea, and reduction of nitrate were determined according to the methodology described by Smibert and Krieg (1994) except that media contained 2.5% (w/v) final salt concentration. For the luminescence test, cultures were grown separately on agar plates with media composition described by Baumann and Baumann (1981) and were examined periodically for 12–36 h in dark. Growth at 10–45 °C was determined in trypticase soy broth (TSB) (Difco) containing 2.5% NaCl (w/v) for 72 h at pH 8. The requirement of NaCl for growth was determined in artificial seawater broth containing 0–7% NaCl at 28 °C for 72 h (Deep et al. 2014). In addition to classical tests, phenotypic characterizations were also carried out using API 50 CH (bioMérieux) and Biolog GNII microplate according to manufacturers' instruction with minor modification (Kumari et al. 2014a). Constitutive enzymes productions were tested using API ZYM (bioMérieux). The result obtained after Biolog and API 50 CH test were coded for numerical analysis in binary format and the dendrogram was drawn using the XLSTAT software package (version 2015.4.01.20; Addinsoft, Paris France) using the Agglomerative Hierarchical Clustering (AHC) method, calculated using the Jaccard coefficient and UPGA (unweighted pair-group average) linkage.

Antimicrobial susceptibility testing

Susceptibility of the strains towards different antimicrobials was examined on MA following the Kirby-Bauer disc diffusion method, as described by the Clinical and Laboratory Standards Institute, USA (Deng et al. 2020). 12 different antimicrobials discs (Hi-Media, India) namely tetracycline (30 µg), trimethoprim (5 µg), streptomycin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), vancomycin (30 µg),

nalidixic acid (30 µg), ampicillin (10 µg), sulfamethoxazole-trimethoprim (25 µg), kanamycin (30 µg), rifampicin (5 µg) and ciprofloxacin (5 µg) were used in this study. In brief, the cells were grown for 18 h in marine broth, and the turbidity of cells was adjusted to an optical density (OD) 0.5 McFarland using the sterile marine broth. 100 µl of this cell suspension was spread onto the agar plates after which the discs were placed on it and the plates were incubated at 28 °C for 24 h. Susceptibility of the strains to each antimicrobial was recorded by measuring the mean inhibition zone diameter. The diameter of each antimicrobial disc was 6 mm. Strains were considered susceptible when the inhibition zone diameter was greater than 8 mm (Kumari et al. 2016). All the experiments were performed in triplicates.

16S rRNA gene amplification, sequencing and analysis

DNA was isolated according to a standard protocol and used as a template for PCR amplification. 16S rRNA gene was amplified by PCR using a primer pair (27F, 5'-GAGTTT GATCCTGGCTCAG-3' and 1525R, 5'-AAAGGAGGTGAT CCAGCC-3') and protocol as described earlier (Kumari et al. 2014a). PCR amplicons of the expected size were gel purified and sequenced using 8 capillary genetic analyzers (model number 3500, Applied Biosystems) following the manufacturer's protocol. The nucleotide sequences obtained were assembled using the sequence alignment editor program BioEdit (Hall 2007) and were compared with those in GenBank after BLAST searches (Altschul et al. 1997) and using the EzTaxon-e server (Kim et al. 2012) for identification of *Vibrios*. Finally, a neighbor-joining phylogenetic tree was constructed with 16S rRNA sequences of the *Vibrio* strains along with type species using MEGA 5 (Tamura et al. 2011).

Multilocus sequence analysis (MLSA)

Multilocus sequence analysis was performed to elucidate the phylogenetic position of the *Vibrio* strains isolated in this study. Primers for amplification of eight housekeeping genes namely *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA* are presented in Table 2. PCR conditions were followed as described earlier (Kumari et al. 2014a). PCR products of the expected size were gel-purified by NucleoSpin Gel & PCR Clean-up kit (Macherey–Nagel, Germany) and sequenced as described earlier (Kumari et al. 2014a). The nucleotide sequences were compared with those in GenBank using the nucleotide BLAST algorithm (Altschul et al. 1997) following the nucleotide collection (nr/nt) database and optimized for highly similar sequences (mega blast). Sequences were aligned by default parameters of the Clustal program of MEGA5, refined manually, and trimmed to a common

Table 2 List of primers used in MLSA

	Gene	Primer name	Sequence (5' → 3')	Annealing temperature (°C) used in this study
1	<i>gapA</i> *	VgapA150F	AACTCACGGTCGTTT CAA A	47
		VgapA899R	CGTTGTCGTACCAAGATAC	
2	<i>ftsZ</i> *	VftsZ75F	GCTGTTGAACACATGGTACG	49
		VftsZ800R	GCACCAGCAAGATCGATATC	
3	<i>ftsZ</i> *	VftsZ75F	GCTGTTGAACACATGGTACG	50
		VftsZ700R	ATCATGGCGTGACCCATTTC	
4	<i>topA</i> *	VtopA400F	GAGATCATCGGTGGTGAT G	51
		VtopA1200R	GAAGGACGAATCGCTTCGTG	
5	<i>mreB</i> *	VmreB12F	ACTTCGTGGCATGTTTTTC	46
		VmreB999R	CCGTGCATATCGATCATTTTC	
6	<i>pyrH</i> *	VpyrH80F	GATCGTATGGCTCAAGAAG	48
		VpyrH503R	TAGGCATTTTGTGGTCACG	
7	<i>rpoA</i> *	rpoA-01-F	ATGCAGGGTCTGTDACAG	53
		rpoA-03-R	GCGGCCACTTTTCTAGACGC	
8	<i>recA</i> #	drecA-F	GATGCAGAGCACGCACTAGA	52
		drecA-R	ATGGTGCAGCAATCTTGTTTC	
9	<i>recA</i> #	drecA2-F	TGTGCGTTTATTGATGCAGA	48
		drecA2-R	GTAGTCGTTTCTGGGTACC	
10	<i>gyrB</i> #	gyrB-F	GTAGTAAACGCTCTGTCCGA	49
		gyrB-R	GGGTTCTCAACTAGGAACTC	
11	<i>gyrB</i> *	VgyrB274F	GAAGTTATCATGACGGTACTTC	45
		VgyrB1171AR	CCTTTACGACGAGTCATTTC	

Genes used for MLSA analysis: recombination repair protein (*recA*), uridylyate kinase (*pyrH*), glyceraldehyde 3-phosphate dehydrogenase (*gapA*), rod-shaping protein gene B subunit (*mreB*), DNA gyrase B subunit (*gyrB*), and topoisomerase I (*topA*), cell division protein (*ftsZ*), RNA polymerase alpha-subunit (*rpoA*)

Primers were * adopted from Sawabe et al. (2007); # designed in this study

length. Multilocus gene phylogeny was prepared with concatenated sequences of eight protein-coding genes of *Vibrio* strains and type species following neighbour-joining analysis using MEGA 5.

Pulsed-field gel electrophoresis (PFGE)

Vibrio strains were grown freshly in TSB media supplemented with 2.5% NaCl for 12–14 h at 28 °C. Cells were harvested and dissolved in 1 × TE buffer. Cell suspension (optical density adjusted to ~0.5 McFarland) was prepared for making plugs. 20 µl of proteinase K (20 mg/ml) and 10 µl (10 mg/ml) of lysozyme were added to 300 µl of cell suspension in the Eppendorf tube. 1.2% agarose (InCert-Agarose, Lonza) dissolved in 1X TE and, equilibrated at 54 °C was added to the cell suspension in a 1:1 ratio and, were dispensed into the disposable plug molds (Bio-Rad) after gentle mixing (three plugs were made for each strain). The plugs were kept at 4 °C for 15–20 min. The plugs were transferred to cell lysis buffer (50 mM Tris: 50 mM EDTA pH 8 + 1% sarcosine) along with 10 µl proteinase K and 10 µl of lysozyme and were incubated for 2–6 h at 54–55 °C for in-gel lysis. The treated plugs were

washed with sterile Milli Q thrice at 54 °C with gentle shaking followed by 3–4 times washing with 1 × TE. Plugs were then stored at 4 °C for further use.

Restriction enzyme *NotI* (Thermo scientific) was used for the digestion of DNA. Each plug was treated with the digestion equilibration mixture (Buffer O, 20 µl and Milli-Q water, 100 µl) for 1 h at 37 °C followed by overnight digestion at 37 °C in 200 µl of digestion mixture (Buffer O, 20 µl; Milli-Q water, 176 µl and 40 U of *NotI* enzyme). After digestion, plugs were subjected to pulsed-field gel electrophoresis in 0.5 × TBE (Tris–Borate EDTA) buffer in CHEF Mapper (Bio-Rad). The plugs were embedded in 1% agarose gel and restriction fragments were resolved in 0.5 × TBE with running conditions: 6 V/cm with an angle of 120 ° at 14 °C with a switch time of 2–10 s for 13 h and 20–25 s for 6 h. After the complete run, the gel was stained with ethidium bromide solution for 30 min followed by destaining in Milli-Q water for 60 min. Banding pattern in the gel was documented with the Chemi-Doc™ XRST imaging system (Bio-Rad) and was analyzed through Bio-Numeric 7 software (Applied Maths) using the dice coefficient for band-based similarity analysis and UPGMA for cluster analysis.

Nucleotide sequence accession numbers

All sequences are deposited in the NCBI GenBank under accession numbers as follows: KM216222-KM216248, JQ409371-JQ409374, JQ409380, JQ409382-JQ409387 for 16S rRNA; KP299883-KP299919 for *gyrB*; KM884834-KM884870, KJ549658 for *mreB*; KM487612-KM487647, KM487648, KJ549661 for *topA*; KP299921-KP299958 for *ftsZ*; KM921879-KM921915 for *gapA*; KM280783-KM280820 for *recA*; KM280745-KM280782 for *rpoA* and KM280708-KM280744 for *pyrH*. Sequence accession numbers for housekeeping genes and 16S rRNA used in the different analyses in this study are presented in Table S3-S4.

Results

Isolation and phenotypic characterization

A total of 38 different strains belonging to the genus *Vibrio* were identified (Table S1). Except for the strain W9 that was isolated from the seawater sample, the rest of the strains were associated with marine invertebrate i.e. coral and lobster. *Vibrio* strains were obtained from surface mucus layers of five coral species *Porites porites* (9), *Goniastrea retiformis* (3), *Pocillopora verrucosa* (1), *Fungia danai* (6), *Fungia echinata* (11) and, eggs of lobster *Panulirus penicillatus* (7),

the numbers of strains are shown in parenthesis. Information on the geographic locations, marine environmental physical conditions, and the strains isolated from different sources are presented in Table 1. All the strains developed white to creamish, smooth surface, non-luminescent colonies of different sizes on the MA plate. Morphologically, strains were Gram-negative, curved rods. Physiologically, the majority of strains grew between 10 °C and 45 °C and optimally at 28 °C. Most of the strains required NaCl for growth whereas strain S1-14, S1-15, AN11, and AN14 grew in the absence of NaCl. The majority of strains grew in the presence of 7% NaCl. None of the strains were luminescent. All strains were negative for arginine dihydrolase (ADH) activity. Likewise, most of the strains were negative for lysine decarboxylase activity except strain AN43, and AN 72 that were isolated from coral *Fungia echinata*. In contrast, mixed responses were obtained for ornithine decarboxylase activity. Differential phenotypic features among the strains are presented in table S2.

In the Agglomerative Hierarchical Clustering dendrogram prepared using the phenotypic traits, two different observations were recorded (Fig. 1). In one observation, strains representing the same species formed clusters indicating phenotypic uniformity among strains of the same species. For example, strain S1-18 and S1-20 representing *V. neptunius* formed a separate cluster and exhibited close similarity with strain AN38 and AN62 representing *V. nereis* and

Fig. 1 Dendrogram prepared using Agglomerative Hierarchical Clustering (AHC) showing cluster analysis of co-occurrence of phenotypically similar traits among 38 *Vibrio* strains. Strain AN12, AN43 and AN43 did not form cluster with other strains that formed three different clusters

V. ponticus, respectively. Similar observations were also recorded for the majority of strains representing *V. fortis*. On the second observation, strains representing the same species were also found to share different clusters indicating intraspecies phenotypic variability. For example, 8 strains representing *V. fortis* formed three different clusters. Strain AN12 and AN43 representing *V. tubiashii* and *A. fischeri* did not show any phenotypic similarity with other strains and remained as outgroup in the dendrogram.

16S rRNA based phylogenetic analysis

Based on 16S rRNA sequencing and subsequent BLAST analysis using EzTaxon-e indicated that all strains shared more than 97% sequence homology with different type strains of the genus *Vibrio* (Table S1). Out of 7 strains isolated from the eggs of lobsters, a predominance of strains sharing more than 99% similarity with *Vibrio rotiferianus* (strain LBS1 and LBS12) and *Vibrio communis* (strain LBS4 and LBS9) was established. Strain LBS3 shared only 97.36% similarity with *Vibrio fortis* LMG 21557^T. From mucus of coral *Porites porites*, species representing *Vibrio fortis*, *Vibrio diabolicus*, *Vibrio neptunius* were found predominant. *Vibrio inhibens* was found abundant in coral *Goniastrea retiformis*. *Vibrio fortis* was abundant in coral *Fungia danai*. *Fungia echinata* harbored a variety of species with an abundance of *Vibrio alginolyticus* and *Vibrio communis*. In the neighbor-joining phylogenetic tree prepared using the type species of the genus *Vibrio*, the strains formed 10 different clusters (Fig. 2).

MLSA analysis and distribution of strains into clades

An extensive MLSA study with concatenated sequences of 8 housekeeping genes was performed to place the strains to already reported *Vibrio* clades (Sawabe et al. 2013). All strains were distributed in 6 reported clades i.e. *Harveyi*, *Ponticus*, *Nereis*, *Orientalis*, *Splendidus*, and *Mediterranei* (Fig. 3). 47% of total strains belonged to clade *Harveyi* and contained strains representing *V. rotiferianus*, *V. alginolyticus*, *V. owensii*, *V. communis*, *V. azureus*, and *V. harveyi* where *V. alginolyticus* was predominant. 21% of strains represented clade *Splendidus* where all strains had the closest 16S rRNA similarity with *V. fortis*. Clade *Nereis*, *Ponticus*, and *Orientalis* contained single isolate i.e. *Vibrio nereis* strain AN38, *Vibrio ponticus* strain AN62, and *Vibrio tubiashii* strain AN12, respectively.

In terms of association of isolation sources with the clade distribution, it was noted that the strains isolated from coral *Goniastrea retiformis*, *Pocillopora verrucosa*, and seawater belonged to clade *Harveyi*. Except for *Vibrio fortis* strain LBS3 that belongs to the clade *Splendidus*, all strains obtained from eggs of lobster represented clade

Harveyi. Coral *Porites porites* exhibited an abundance of species belonging to the clade *Harveyi* whereas coral *Fungia danai* had an abundance of species belonging to the clade *Splendidus*. No such association was found in coral *Fungia echinata* where strains were distributed in different clades.

Pulsed-field gel electrophoresis (PFGE) analysis

PFGE analysis to determine the intraspecies genetic heterogeneity revealed the distribution of the strains into five major clusters (Fig. 4). However, four strains namely strain LBS1, LBS11, LBS12, and AN62 did not produce banding patterns by PFGE even with the addition of thio-urea. Two major clusters in which one cluster containing AN 43, LBS11, AN62, LBS12, LBS1 revealed significant genetic diversity with the other strains. Notable here that the strains showing 16S rRNA similarity with a particular type species were not genetically identical rather occupy different positions in the PFGE profile. Similarly, strains showing maximum similarity in the MLSA clade profile produced different banding patterns.

Antimicrobial susceptibility

All strains were susceptible to chloramphenicol and more than 60% of strains were susceptible to ciprofloxacin, nalidixic acid, and rifampicin. On the other hand, all strains (100%) were resistant to vancomycin, streptomycin, and sulfamethoxazole-trimethoprim. The majority of strains were resistant to tetracycline (94.7%) followed by erythromycin (89.5%), and kanamycin (81.6%). The frequency of antimicrobial susceptibility of the strains and the response of individual strain to different antimicrobial agents is presented in Table 3. Resistance to different antimicrobials also varied depending on the isolation source of the strains. Strain S6-61 and W9 isolated from coral *Pocillopora* and seawater, respectively, were resistant to 11 out of 12 antimicrobials tested in this study. Resistance to 6, 5, and 4 different antimicrobials was observed from all strains isolated from coral *Fungia danai*, *Goniastrea*, and *Porites*, respectively. Likewise, all strains isolated from the eggs of lobster were resistant to 5 antimicrobials namely trimethoprim, erythromycin, vancomycin, streptomycin, and sulfamethoxazole. A lesser resistance profile was noted from the strains obtained from coral *Fungia echinata* where all strains were resistant to the common three antimicrobials only. However, the majority of the strains were resistant to tetracycline (except strain AN12), erythromycin (except strain AN12 and AN82); kanamycin (except strain AN11 and AN61).

Fig. 2 16S rRNA-based neighbour-joining phylogenetic tree showing the phylogenetic relationship of the isolated *Vibrio* strains with the type species of the genus *Vibrio*. Bootstrap values (based on

1000 replicates) are given at branch points. 16S rRNA gene sequence accession numbers are given in parentheses

Discussion

Diversity analysis of marine *Vibrio* is a dynamic field of investigation. In addition to the possible identification of novel species, it has provided a greater insight into a marine microbial habitat, health, and Spatio-temporal observation that differs across regions. Even after the advent of non-culturable metagenomic and whole-genome sequencing that are more technically complex (Gevers et al 2005), the

majority of the *Vibrio* diversity analysis relies on traditional culturable approaches where individual *Vibrio* strains are taxonomically characterized. However, compared to other bacterial genera taxonomic assignment of *Vibri*os is puzzling due to the remarkable intraspecific diversity of strains of the genus *Vibrio* collected from different habitats (Hoi et al. 1998; Tamplin et al. 1996). Identification of *Vibri*os based on 16S rRNA sequence phylogeny alone is difficult because some of the strains share almost 100% sequence

Fig. 3 Neighbour-joining phylogenetic tree of *Vibrio* strains based on concatenated sequences of eight housekeeping genes. Only bootstrap values above 65% are shown (1000 replications) at branches points.

Bar, 0.02 expected nucleotide substitutions per site. *P. lutimaris* was used as an outgroup. Distribution of the strains in the reported clade are shown in different shaded colour

Fig. 4 Dendrogram showing the clustering of PFGE patterns (digested with *NotI* restriction enzyme) for *Vibrio* strains. The PFGE banding patterns were analyzed with computer software, BioNumerics version 6.1 and the dendrogram was produced using the Dice coefficient and UPGMA algorithm

similarity although represent different species (Thompson et al. 2007). For such, different molecular approaches like multilocus sequence analysis (MLSA) (Sawabe et al. 2007), pulsed-field gel electrophoresis (PFGE) (Banerjee et al. 2014), has been used in combination to maximize the inter-specific resolution.

Species abundance and community composition of marine *Vibrios* is influenced by the abundance of host cell surfaces, hydrologic (temperature, pH, salinity, etc.), and nutrient factors, both organic and inorganic. In these, sea surface temperature has been strongly correlated with the increased abundance of *Vibrios* (Takemura et al. 2014) wherein it was also noted that virulence genes including host cell lysis, bleaching, antimicrobial resistance, and motility in *Vibrios* become unregulated at a threshold temperature above 27 °C. As such, marine *Vibrios* are considered as the indicator of climate change, especially global warming and marine health (Kimes et al. 2011). Climate change has adversely affecting sea surface temperature worldwide (Vezzulli et al. 2013) wherein this region of the Andaman Sea has recorded some major bleaching events in the coral reef due to the rise of sea temperature (Ravindran et al. 1999; Arthur 2000). In view, this current study hypothesis was that abundances of antimicrobial resistance *Vibrio* associated with invertebrate hosts may be observed and the objective being deciphering the diversity and extent of resistance phenotypes of these strains to different antimicrobials.

Culturable isolation of 38 *Vibrio* strains associated with the hosts and seawater supported the hypothesis and also agreed upon the published observation on the principle role of temperature to modulate *Vibrio* communities in the sea. The strains were taxonomically determined following the combination of phenotypic, phylogenetic, and other genetic studies in line with the recommended practices (Sawabe

et al. 2007). Phenotypically the strains had features that are common to the genus *Vibrio*. All the strains have no arginine dihydrolase activity, a signature feature that was considered as an essential character for speciation of the genus *Vibrio* (Baumann et al. 1984). Further, the strains exhibited diverse nutritional requirements as observed in individual tests, and the Agglomerative Hierarchical Clustering dendrogram supported intraspecies phenotypic variability as a function of nutritional diversity for this genus (Thompson and Polz 2006).

16S rRNA analysis allows preliminary species-level identification of a new isolate and also determines the possibility of the novelty of the strains based on the sequence similarity values obtained after BLAST. All the strains shared almost 99% or above 16S rRNA similarities with the type strains of the genus *Vibrio* except one, strain LBS3. It shared only 97.3% similarity with *Vibrio fortis* LMG 21557^T that may indicate the possibility of a novel strain, investigation to which is beyond scope of the current study. The 38 strains represented 14 different species wherein the most abundant species was *Vibrio fortis* followed by *Vibrio alginolyticus*. The type species *Vibrio fortis* LMG 21557^T (Thompson et al. 2003) and *Vibrio alginolyticus* NBRC 15630^T (Sakazaki 1968) were isolated from the white shrimp and spoiled horse mackerel, respectively. Both strains are pathogenic to marine hosts and humans (Wang et al. 2016). The predominance of these strain in the marine environment, therefore, could be correlated with the poor health of the marine invertebrates and also present an immediate threat to local public health safety who earn their livelihood in the region.

In the phylogenetic tree based on concatenated sequences of eight housekeeping genes, significant phylogenetic separation of strains into clades were observed that further supports earlier observations made by Sawabe et al. (2013).

Table 3 Susceptibility of 38 *Vibrio* isolates towards different antimicrobial agents

S. No	Bacterial strains	1	2	3	4	5	6	7	8	9	10	11	12
1	<i>Vibrio</i> species strain LBS1	+	+	+	–	+	+	–	+	+	+	–	–
2	<i>Vibrio</i> species strain LBS3	+	+	+	–	+	+	–	+	+	+	–	–
3	<i>Vibrio</i> species strain LBS4	+	+	+	–	+	+	–	+	+	+	–	–
4	<i>Vibrio</i> species strain LBS6	+	+	+	–	+	+	–	–	+	–	+	–
5	<i>Vibrio</i> species strain LBS9	+	+	+	–	+	+	–	+	+	+	–	–
6	<i>Vibrio</i> species strain LBS11	+	+	+	–	+	+	–	+	+	+	–	–
7	<i>Vibrio</i> species strain LBS12	–	+	+	–	+	+	–	+	+	–	–	–
8	<i>Vibrio</i> species strain S1-10	+	–	+	–	+	+	–	–	+	+	+	+
9	<i>Vibrio</i> species strain S1-11	+	+	+	–	+	+	–	+	+	+	–	+
10	<i>Vibrio</i> species strain S1-14	+	+	+	–	+	+	–	+	+	+	+	–
11	<i>Vibrio</i> species strain S1-15	+	+	+	–	+	+	+	+	+	–	+	–
12	<i>Vibrio</i> species strain S1-17	+	–	+	–	+	+	–	–	+	+	+	+
13	<i>Vibrio</i> species strain S1-18	+	+	+	–	+	+	–	+	+	+	–	+
14	<i>Vibrio</i> species strain S1-20	+	–	+	–	–	+	–	+	+	+	–	+
15	<i>Vibrio</i> species strain S2-24	+	+	+	–	+	+	+	+	+	+	+	–
16	<i>Vibrio</i> species strain S2-25	+	+	+	–	–	+	+	+	+	–	–	–
17	<i>Vibrio</i> species strain S2-26	+	+	+	–	+	+	+	+	+	+	–	–
18	<i>Vibrio</i> species strain S3-31	+	+	+	–	+	+	–	–	+	–	+	–
19	<i>Vibrio</i> species strain S3-32	+	+	+	–	+	+	–	+	+	+	–	–
20	<i>Vibrio</i> species strain S6-61	+	+	+	–	+	+	+	+	+	+	+	+
21	<i>Vibrio</i> species strain S7-71	+	+	+	–	+	+	+	+	+	+	–	+
22	<i>Vibrio</i> species strain S7-72	+	–	+	–	+	+	+	+	+	+	+	–
23	<i>Vibrio</i> species strain S7-73	+	–	+	–	+	+	–	–	+	+	+	–
24	<i>Vibrio</i> species strain S7-74 A	+	–	+	–	+	+	–	–	+	+	+	+
25	<i>Vibrio</i> species strain S7-74 B	+	+	+	–	+	+	–	+	+	+	+	–
26	<i>Vibrio</i> species strain S7-77	+	–	+	–	+	+	–	–	+	+	–	–
27	<i>Vibrio</i> species strain W9	+	+	+	–	+	+	+	+	+	+	+	+
28	<i>Vibrio</i> species strain AN11	+	+	+	–	+	+	–	+	+	–	–	–
29	<i>Vibrio</i> species strain AN12	–	+	+	–	–	+	–	–	+	+	–	–
30	<i>Vibrio</i> species strain AN14	+	+	+	–	+	+	–	+	+	+	–	–
31	<i>Vibrio</i> species strain AN38	+	+	+	–	+	+	–	–	+	+	–	–
32	<i>Vibrio</i> species strain AN43	+	–	+	–	+	+	+	+	+	+	–	–
33	<i>Vibrio</i> species strain AN60	+	–	+	–	+	+	+	–	+	+	+	+
34	<i>Vibrio</i> species strain AN61	+	+	+	–	+	+	–	+	+	–	–	+
35	<i>Vibrio</i> species strain AN62	+	+	+	–	+	+	–	–	+	+	–	–
36	<i>Vibrio</i> species strain AN72	+	+	+	–	+	+	+	+	+	+	–	–
37	<i>Vibrio</i> species strain AN81	+	+	+	–	+	+	+	+	+	+	–	+
38	<i>Vibrio</i> species strain AN82	+	–	+	–	–	+	+	+	+	+	–	+

+, resistant, –, susceptible

1, Tetracycline (30 µg); 2, Trimethoprim (5 µg); 3, Streptomycin (10 µg); 4, Chloramphenicol (30 µg); 5, Erythromycin (15 µg); 6, Vancomycin (30 µg); 7, Nalidixic acid (30 µg); 8, Ampicillin (10 µg); 9, Sulfamethoxazole-Trimethoprim (23.75/1.25 µg); 10, Kanamycin (30 µg); 11, Rifampicin (5 µg); 12, Ciprofloxacin (5 µg)

Even with the addition of the strains in this study, distinct phylogenetic separation of clade *Ponticus*, *Scophthalmi*, *Nereis*, *Orientalis*, and *Mediterranei* were observed. But, clade *Harveyi* and *Splendidus* were found to be more evolutionary divergent as several independent clusters were obtained. With the incorporation of more novel species in the genus *Vibrio*, it might be possible to further revisit the

clade *Harveyi* and *Splendidus* for more phylogenetic robustness. This observation is in good agreement of the conclusions made earlier in different studies (Thompson et al. 2007; Sawabe et al. 2007, 2013).

PFGE analysis is the most widely used approach used for determining genetic diversity in *Vibrio* species (Rahaman et al. 2015). PFGE analysis retains the structural integrity

of chromosome and is considered the gold standard whole-genome profiling based on more discriminative restriction enzyme cleavage patterns among strains of the same species (Rahaman et al. 2015). The PFGE dendrogram resulted in different banding patterns and so the differential clustering of the same species that indicate intrinsic genomic diversities among the species. The observation is in the agreement of previous reports that suggest distinct genomic diversities among strains of a single species of *Vibrio* (Suffredini et al. 2011).

Resistance to these large number of antimicrobials by the *Vibrio* strains may be considered a noble observation. It is in contrast to many studies where *Vibrio* sp. were found to be highly susceptible to most antibiotics (Mala et al. 2014). Development of resistance to a wide range of antimicrobials by marine *Vibrios* may be correlated with genome functional changes due to a rise in sea surface temperature, excessive anthropogenic use of antibiotics that is ending up in seawater, and catalyzing resistance development in the marine microbes (Letchumanan et al. 2015). This antimicrobial resistance profile in a combination of the fact that predominant strains are pathogenic to marine and human life may be regarded as a potential health and environmental safety threat.

Conclusions

This study has analyzed the *Vibrio* diversity associated with the marine *Vibrio* strains isolated from invertebrate hosts obtained from the Andaman Sea and their resistance to different antimicrobial agents. Extensive phenotypic, phylogenetic and molecular analysis based on PFGE and MLSA has indicated the strains possess remarkable nutritional diversity, interspecies variability, genomic diversity, and diverse evolutionary origin. Considering the over the threshold sea temperature of the Andaman Sea (27–29 °C), the predominance of pathogenic strains like *Vibrio fortis* and *Vibrio alginolyticus*, and multidrug resistance phenotype, this report points out imminent health and environmental safety risks to the marine as well as human life in that region. Any further rise of sea surface temperature will surely promote the proliferation of the pathogenic *Vibrio* that not only replace the natural marine microflora rather will increase the risks to many fold. As such, there is a need for constant surveillance of abundance, community structure, and drug resistance profiles of the *Vibrios* in this region. Thus, a strategy could be developed both at the scientific and policy level to ensure effective management of the imminent environmental and health risks in an ordered and timely fashion.

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Compliance with ethical standards

Conflict of interest No conflict of interest exists.

Ethical approval This article does not contain any studies with human participants and/or animals performed by any of the authors. The formal consent is not required in this study.

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