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# Genomic insights into antibiotic-resistant *Vibrio* species from clinical and coastal environmental sources in India

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ABSTRACT

The occurrence and impact of pathogenic *Vibrio* sp. in coastal waters are strongly influenced by climate change indicators such as ocean warming, sea-level rise, and extreme weather events. This study aimed to compare the virulence and antimicrobial resistance (AMR) profiles of *Vibrio cholerae* from clinical and environmental sources across India's coastal regions. We also examined pathogenic traits in other marine *Vibrio* sp. We hypothesized that *Vibrio* spp. from different environments would show distinct virulence and AMR patterns shaped by their ecological context. To investigate this, we conducted antimicrobial susceptibility testing and whole-genome sequencing (WGS) on both clinical and environmental isolates. Our findings reveal that environmental *V. cholerae* from coastal waters possess genes promoting host adhesion and haemolytic activity. Similarly, *Vibrio alginolyticus* and *Vibrio vulnificus* harboured virulence factors aiding tissue attachment and invasion. Resistance profiling showed environmental *V. cholerae* were resistant to fluoroquinolones and macrolides, while clinical isolates were resistant to aminoglycosides and sulphonamides. The presence of antibiotic-resistant *Vibrio* in marine environments poses a significant public health risk, especially given frequent human interactions with coastal waters for recreation, fishing, and transport. Climate change may exacerbate the proliferation and movement of these pathogens across aquatic and terrestrial systems, increasing the likelihood of human exposure. Moreover, the potential for horizontal gene transfer of resistance genes among pathogenic marine bacteria further highlights the need for surveillance and mitigation strategies to address the growing threat of AMR in marine ecosystems.

## 1. Introduction

Over 200 species of *Vibrio* have been identified within the aquatic environment, with at least a dozen known to cause diseases in humans and marine organisms. Among these, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus* are frequently found in coastal waters and are associated with water-associated diseases, with *V. cholerae* being particularly notorious (Abdulaziz et al., 2023; Arunkumar et al., 2020). The spatially heterogeneous distribution of *Vibrio* spp. is significantly influenced by hydroclimatic and anthropogenic factors, with notable prevalence in regions such as the Bay of Bengal (India and Bangladesh), the coastal areas of Latin America, and the Baltic Sea in Northern Europe (Baker-Austin et al., 2013; Lipp et al., 2002; Mutreja et al., 2011). Diarrheal diseases associated with these organisms are primarily transmitted through the consumption of contaminated water and food (Baker-Austin et al., 2018). A recent report by the World Health Organisation (WHO) indicated that nearly 70 % of the transmission of diarrheal diseases could be eradicated through the implementation of Water, Sanitation, and Hygiene (WaSH) protocols (WHO, 2023). The remaining disease burden may result from additional exposure routes, including environment-to-human transmission during activities such as bathing, washing, or fishing. In coastal regions where

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WaSH infrastructure is frequently inadequate and *V. cholerae* is endemic, the convergence of environmental and infrastructural risk factors substantially increases the likelihood of diarrheal disease outbreaks (Ashrafuzzaman et al., 2023; Susilawati et al., 2022; Lipp et al., 2002).

Extreme weather events, such as floods, landslides, and cyclones, can severely damage the WaSH infrastructure, leading to the direct mixing of sewage with coastal waters, which increases the chances of environment-to-human transmission of pathogens (McKenzie et al., 2021; Rahman and Rahman, 2015). Documented instances of such transmission include the surge in diarrhoeal and gastroenteric illnesses in Louisiana and Mississippi following Hurricane Katrina in 2005 (Illnesses, 2005), primarily attributed to overcrowded shelters and compromised sanitation systems. Similarly, in Kerala, the 2018 catastrophic flood was followed by a marked increase in acute diarrheal disease, linked to the contamination of drinking water sources and inadequate disinfection (Anas et al., 2023; Shankar et al., 2021). The pathogenic bacteria infiltrating coastal waters from septic sewage may harbour antibiotic-resistance genes (Fonti et al., 2021), compounding their present challenges. Another key climate change indicator influencing the distribution of *Vibrio* species in subtropical and temperate regions is global warming (Baker-Austin et al., 2017; Baker-Austin et al., 2016; Baker-Austin et al., 2013). The increasing frequency and intensity of marine heatwaves prolong periods of elevated sea surface temperatures, particularly in coastal zones, creating favourable conditions for the proliferation of *Vibrio* spp. (Baker-Austin et al., 2016; Takemura et al., 2014). These bacteria thrive in warm, low-salinity waters, and their abundance is strongly correlated with sea surface temperatures exceeding 18 °C (Takemura et al., 2014). As a result, human exposure to such waters, whether through recreational activities, consumption of contaminated seafood, or flood-related contact, can lead to an increase in *Vibrio*-associated illnesses. Recent global assessments estimate that the coastal area environmentally suitable for *Vibrio* transmission has expanded by approximately 329 km per year since 1982, reflecting a significant poleward shift in risk zones (Romanello et al., 2023).

Comprehensive and multidisciplinary strategies are being discussed globally to interrupt the transmission pathways of pathogens from the environment to humans. These strategies incorporate several pivotal elements. Firstly, they entail the development of risk maps for contaminated regions, which are constructed based on in-situ observations and remote sensing data (Anas et al., 2021; Racault et al., 2019). Secondly, they incorporate citizen science initiatives and awareness campaigns (George et al., 2021). These initiatives are vital for fostering public involvement and disseminating knowledge. Thirdly, they employ digital tools and predictive models indispensable for data interpretation and future projections (Sathyendranath et al., 2020). The surveillance of the environmental distribution of water-associated pathogens in coastal waters, along with the genomic comparison of these isolates, lays the groundwork for this comprehensive approach.

Despite their clinical and environmental importance, *Vibrio* species remain an insufficiently monitored public health threat in India, attributable to deficiencies in surveillance data and insufficient efforts to correlate the ecological distribution of these pathogens with disease incidence (Chatterjee et al., 2020). This situation is further complicated by the ability of these microorganisms to adapt and persist in various aquatic environments, alongside the impacts induced by climate change, urbanization, and other anthropogenic factors (El-Sayed and Kamel, 2020; Usmani et al., 2021) (Brumfield et al., 2021). Research conducted in developed nations frequently investigates the ecology, resistance patterns, and climate-driven changes related to *Vibrio* species, offering valuable insights that are often absent in the Indian context (Brumfield et al., 2023; Deeb et al., 2018; Vaiyapuri et al., 2021). The present study aims to address this gap by conducting a comprehensive analysis of the genomes of clinical and environmental isolates of *Vibrio cholerae*, as well as environmental isolates of *V. alginolyticus* and *V. vulnificus*, sourced from the coastal regions of India.

## 2. Materials and methods

### 2.1. Isolation and antibiotic susceptibility of Vibrio sp.

The present study focuses on *V. cholerae* (6 isolates—including 1 clinical isolate from stool sample of cholera patient, 2 from coastal waters off Chennai [Bay of Bengal] and one each from a groundwater source, Cochin [Arabian Sea], and Vembanad Lake), *V. alginolyticus* (5 isolates obtained from marine litter collected along the beaches of the Lakshadweep archipelago), and *V. vulnificus* (3 isolates—comprising one each from water samples collected from the coastal waters off Chennai [Bay of Bengal] and Cochin [Arabian Sea], and from water filtered for phytoplankton from Vembanad Lake) (Table 1) (Abdulaziz et al., 2023; Krishna et al., 2020). These isolates were selected from a comprehensive collection of 568 *Vibrio* isolates obtained from clinical and marine water samples collected across the Bay of Bengal, Arabian Sea, and Vembanad Lake, India. These isolates were archived in the Marine Microbial Reference Facility (MMRF) maintained at CSIR–National Institute of Oceanography, Regional Centre, Cochin. The isolates of *Vibrio* spp. were retrieved from lyophilised stocks in Luria-Bertani broth, following standard protocols. They were further purified on thiosulfate citrate bile salt sucrose (TCBS) agar plates with overnight incubation at 28 °C (Anas et al., 2021). The pure colonies, exhibiting morphological characteristics of *Vibrio* species, were transferred to Luria-Bertani agar slants and maintained through regular subculturing. The identity of isolates was confirmed by sequencing the 16S rRNA gene following the primer combinations and PCR (Polymerase Chain Reaction) conditions described in our previous article (Abdulaziz et al., 2023).

To understand the antimicrobial resistance pattern of *Vibrio* spp. Kirby-Bauer disc diffusion technique was employed with a set of 16 antibiotics that fall under different groups of antibiotics. The antibiotic discs (HiMedia, India) were systematically positioned on Müller-Hinton agar plates that was inoculated with 12 h old bacterial broth culture, utilizing a sterile cotton swab. Following a 24-hour incubation period at 28 °C (for environmental *Vibrio*) and 37 °C (for clinical strain), the zone of inhibition surrounding each antibiotic disc was measured from each plate. The sensitivity to each antibiotic was assessed following the standard antimicrobial zone size interpretation chart provided by CLSI (2021). Resistance to antibiotics including ampicillin 25 μg, cefalexin 30 μg, cefoxitin 30 μg, tetracycline 30 μg, ceftriaxone 30 μg, cefepime 30 μg, erythromycin 15 μg, chloramphenicol 30 μg, nalidixic acid 30 μg, norfloxacin 10 μg, gatifloxacin 5 μg, moxifloxacin 5 μg, imipenem 10 μg, gentamicin 10 μg, trimethoprim 10 μg and meropenem 10 μg were evaluated. Multiple antibiotic resistance (MAR) index was calculated by calculating the ratio of the number of antibiotics to which bacterium is resistant, to the total number of antibiotics tested (Krumperman, 1983).

### 2.2. Whole genome sequencing

To understand the genetic basis of antimicrobial resistance and virulence of isolated *Vibrio* sp. and the associated mobile genetic elements, including phages, insertion sequences, transposons and plasmids, we performed whole genome sequencing of bacterial isolates that were resistant to at least one antibiotic using short-read platform Illumina. Genomic DNA was extracted from 24-hour-old cultures of bacteria grown in Luria-Bertani broth using the commercially available DNAzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Whole-genome sequencing was performed using the Illumina HiSeq 4000 platform (2 × 150-bp reads) to generate paired-end reads. Paired-end libraries were prepared from a starting concentration of 100 ng of intact genomic DNA using an Illumina Nextera chemistry kit according to the manufacturer's instructions, with IDT for Illumina 10-bp indices.

**Table 1**
Details of *Vibrio* species for which whole genome sequencing was performed in the current study.

| Sl no. | Sample details | | | Source detail | |
|---|---|---|---|---|---|
| | Species | MMRF ID | NCBI accession number | Geographical location/latitude, longitude | Type of sample |
| 1. | *V. cholerae* | 1099 | PP780018 | Calicut, Kerala/clinical sample | Clinical |
| 2 | *V. cholerae* | 1910 | MW934489 | Chellanam, Kerala/76.273°E, 9.839 °N | Groundwater |
| 3 | *V. cholerae* | 1421 | MZ008383 | Chennai, Tamil Nadu/80.491°E, 12.955°N | Seawater, Bay of Bengal |
| 4 | *V. cholerae* | 1423 | MZ008384 | Chennai, Tamil Nadu/80.491°E, 12.955°N | Sea water, Bay of Bengal |
| 5 | *V. cholerae* | 1432 | MZ008391 | Off Kochi, Kerala/76.173°E, 9.950 °N | Sea water, Arabian Sea |
| 6 | *V. cholerae* | 1896 | MW934488 | Aroor, Kerala/76.317°E, 9.879 °N | Lake water, Vembanad lake |
| 7 | *V. alginolyticus* | 2244 | ON527711 | Lakshadweep/72.194°E, 10.872°N | Marine litter, Arabian Sea |
| 8 | *V. alginolyticus* | 2258 | ON527725 | Lakshadweep/72.194°E, 10.872 °N | Marine litter, Arabian Sea |
| 9 | *V. alginolyticus* | 2259 | ON527726 | Lakshadweep/72.194°E, 10.872 °N | Marine litter, Arabian Sea |
| 10 | *V. alginolyticus* | 2260 | ON527727 | Lakshadweep/72.194°E, 10.872 | Marine litter, Arabian Sea |
| 11 | *V. alginolyticus* | 2262 | ON527729 | Lakshadweep/72.194°E, 10.872 °N | Marine litter, Arabian Sea |
| 12 | *V. vulnificus* | 1440 | MZ008398 | Off Kochi, Kerala/76.090°E, 9.926 °N | Sea water, Arabian Sea |
| 13 | *V. vulnificus* | 1744 | MW934457 | Kochi, Kerala/76.173°E, 9.950 °N | Phytoplankton, Vembanad lake |
| 14 | *V. vulnificus* | 1934 | MW934504 | Chennai, Tamil Nadu/80.309°E, 13.004 °N | Sea water, Bay of Bengal |

### 2.3. Bioinformatic analysis

Raw sequence data was generated and *fastq* files were obtained. The quality of reads was determined using FASTQC (v.0.11.5); low-quality reads were filtered out, and adapter regions were removed using Trim-Galore (version 0.6.5dev). The *fastq* files obtained were assembled de novo using Unicycler (v0.4.8) (Wick et al., 2017), with one round of polishing with Pilon (Walker et al., 2014), and the quality of assembly was assessed using QUAST v5.2.0 (Gurevich et al., 2013). The assembled genomes were annotated using Prokka (Seemann, 2014) and were deposited in the NCBI repository under accession numbers provided in Supplementary file, Table 1. To confirm the findings from 16S rRNA based species identification, in silico DNA-DNA hybridisation was also performed using Genome-to-Genome Distance Calculator (v 3.0) (Meier-Kolthoff et al., 2022).

Phage sequences within the genome were identified using PHASTER (https://phaster.ca/); acquired antibiotic resistance genes and virulent genes were identified using ABRicate (Seemann) with MegaRes (Doster et al., 2019) and VFDB databases (Liu et al., 2021), respectively. Mobile genetic elements were detected using MobileElementFinder (v1.0.3) (https://cge.food.dtu.dk/services/MobileElementFinder/) and genomic islands were compared using Island Compare (Bertelli et al., 2022) with the prediction tool, IslandPath (Bertelli and Brinkman, 2018). For biotyping, identification of pandemic lineage and pathogenicity islands in *V. cholerae* isolates was performed using CholeraeFinder tool (https://cge.cbs.dtu.dk/services/CholeraeFinder). A Sequence Type (ST) is a unique identifier assigned to a bacterial strain based on the combination of alleles at multiple housekeeping gene loci. To determine the sequence type of the isolates, MLST was performed using the schemes available at PubMLST for *V. cholerae* and *V. vulnificus* (Jolley et al., 2018; Seemann, 2014). Core genome alignment was obtained by mapping trimmed reads of *V. cholerae*, *V. vulnificus* and *V. alginolyticus* with reference genome *V. cholerae* V060002 (for non-O1/non-O139, GenBank accession number: NZ_AP018677.1), O1 biovar El Tor strain E7946 (for O1 serotype, GenBank accession number: CP047303.1), *V. vulnificus* NBRC 15645 (GenBank accession number: CP012881.1), and *V. alginolyticus* strain E110 (GenBank accession number: NZ_CP098034.1) respectively, using snippy v4.6.0 (https://github.com/tseemann/snippy), with a minimum coverage of 10, minimum fraction of 0.9 and minimum vcf variant call quality of 100. The SNP distance matrix was obtained using snp-dist (https://github.com/tseemann/snp-dists). Using Gubbins v3.3.0 recombinant regions were identified and removed, and maximum-likelihood phylogenetic tree was constructed from the multi-sequence alignment using RAxML v1.0.1 available in Gubbins (Croucher et al., 2014). The resultant phylogenetic trees and its associated data were visualised using iTOLv6.8.1 (Croucher et al., 2014).

## 3. Results

### 3.1. Antibiotic susceptibility of isolated Vibrio sp.

The absence of an inhibition zone surrounding the disc was used as the criterion to classify a *Vibrio* spp. as AMR. Comparatively, *V. cholerae* isolates exhibited a higher MAR index than *V. alginolyticus* and *V. vulnificus* and were unanimously resistant to erythromycin and ampicillin. Within the isolates of *V. cholerae*, the groundwater isolate (MMRF 1910) and the Vembanad lake isolate (MMRF 1896) showed a low MAR index compared to clinical and other environmental isolates. Except for these, all other isolates were resistant to 50 % of the antibiotics tested in this study. Though resistance to erythromycin and gentamicin was observed in strains of *V. algniolyticus*, resistance was detected in only three isolates. *V. alginolyticus* MMRF2259 was found to be only resistant to tetracycline. *V. vulnificus* isolates exhibited a uniform antimicrobial resistance pattern where all strains were found to be resistant only against tetracycline. Resistance to cephalosporins and carbapenems was detected only in *V. cholerae* strains isolated in this study, suggesting differential gene acquisition. Table 2 provides an overview of phenotypic antibiotic sensitivity.

### 3.2. Genome assembly and annotation

The *V. cholerae* isolates studied here had an average GC content of 47.53 %. In comparison, *V. alginolyticus* and *V. vulnificus* had average GC contents of 44.59 % and 46.87 %, respectively, and the species identities were confirmed with in silico DNA-DNA hybridisation analysis (Supplementary Table 2). The genome size was 3.9 Mb for *V. cholerae* and 4.8 Mb for *V. vulnificus*, while the genome size of *V. alginolyticus* ranged from 5 to 5.2 Mb. Repeat regions were detected in only two genomes of *V. cholerae* (MMRF 1432 and MMRF 1423), and no isolates in this study were found to carry plasmids. An in-depth overview of the assembled and annotated genome is provided in the Supplementary material (Supplementary Table 1). Fig. 1 provides a visual representation of the AMR gene locations, genomic islands, and homology regions within the isolated species. Notably, *V. cholerae* isolated from the Arabian Sea (MMRF1432) and the Bay of Bengal (MMRF1421) both exhibited seven unique genomic islands, predominantly located in the 4 Mbp region, which was notably higher compared to the clinical isolate of *V. cholerae* (MMRF1099) that had only four genomic islands. Among the *V. alginolyticus* isolates, MMRF 2262 had the fewest number of genomic islands (five), while the remaining isolates had 9–11 genomic islands. Furthermore, despite being isolated from different environments, *V. vulnificus* showed the highest genomic homology compared to the other species analysed in the study.

**Table 2**
Antibiotic resistance pattern and MAR index values of *Vibrio* isolates examined in this study.

| Name of the isolate | Antibiotics to which resistance was observed | MAR Index |
|---|---|---|
| *V. cholerae* MMRF 1099 | Cefalexin, cefoxitin, ceftriaxone, cefepime, trimethoprim, ampicillin, imipenem, gentamicin, chloramphenicol | 0.56 |
| *V. cholerae* MMRF 1421 | Cefalexin, cefoxitin, ceftriaxone, cefepime, moxifloaxacin, gatifloxacin, nalidixic acid, ampicillin | 0.50 |
| *V. cholerae* MMRF1423 | Cefalexin, cefoxitin, ceftriaxone, cefepime, moxifloaxacin, gatifloxacin, nalidixic acid, ampicillin | 0.50 |
| *V. cholerae* MMRF 1432 | Cefalexin, cefoxitin, ceftriaxone, cefepime, moxifloaxacin, gatifloxacin, nalidixic acid, ampicillin | 0.50 |
| *V. cholerae* MMRF 1896 | Moxifloaxacin, gatifloxacin, nalidixic acid, ampicillin | 0.25 |
| *V. cholerae* MMRF 1910 | Nalidixic acid, ampicillin | 0.13 |
| *V. alginolyticus* MMRF2244 | Erythromycin, gatifloxacin, moxifloxacin, nalidixic acid | 0.25 |
| *V. alginolyticus* MMRF2258 | Erythromycin, gatifloxacin, moxifloxacin, nalidixic acid | 0.25 |
| *V. alginolyticus* MMRF2259 | Tetracycline | 0.06 |
| *V. alginolyticus* MMRF2260 | Erythromycin, gatifloxacin, moxifloxacin, nalidixic acid | 0.25 |
| *V. alginolyticus* MMRF2262 | Tetracycline | 0.06 |
| *V. vulnificus* MMRF 1440 | Tetracycline | 0.06 |
| *V. vulnificus* MMRF 1744 | Tetracycline | 0.06 |
| *V. vulnificus* MMRF1934 | Tetracycline | 0.06 |

### 3.3. SNP based phylogeny and global comparison of antimicrobial resistance in Vibrio sp.

An SNP-based phylogeny was constructed to examine the relationship between *Vibrio* spp. isolates from this study and reference strains from food, environmental, and clinical sources across diverse geographic regions available in the NCBI repository. Environmental (marine and freshwater) and clinical genomes were analysed separately, given their serotype variability. While O1 and non-O1/non-O139 represent serotypes, they often correspond to distinct phylogenetic lineages: the O1 serogroup is strongly associated with epidemic and pandemic cholera, whereas non-O1 strains exhibit greater genetic diversity and are typically linked to environmental reservoirs. *V. cholerae* isolates from marine and freshwater ecosystems formed distinct clades (Fig. 2A). The isolate from Vembanad Lake (MMRF1896) clustered with clinical isolates from Asian countries. At the same time, those from coastal waters off Kochi and Chennai (MMRF1432, MMRF1423, MMRF1421) formed a separate clade, also showing similarities with Asian clinical strains. Groundwater isolate MMRF1910 aligned with clinical isolates from Asia. Clinical isolates showed clonal expansion of the O1 serotype, with strain MMRF1099 exhibiting minimal SNP variation and clustering with other O1 strains from Asia (Fig. 2B). *V. alginolyticus* strains formed three distinct clades, phylogenetically related to environmental strains from Asia (Fig. 2C). A single clade of *V. vulnificus* showed similarity to foodborne strains from North America (Fig. 2D).

This study also analysed clinical and environmental *V. cholerae* isolates for various resistance genes. The *var*G gene associated with resistance was present in clinical (MMRF1099) and marine (MMRF1421, 1423 and 1432) isolates of *V. cholerae*. The *V. cholerae* isolates from the groundwater (MMRF1910) and Vembanad Lake (MMRF1896), which didn't show the presence of *var*G gene, that conferred resistance to cephalosporins and carbapenems. Additionally, quinolone resistance, mediated by the *qnr*VC gene, was prevalent among marine isolates but notably absent in isolates from groundwater and the clinical isolate. The ubiquitous presence of CRP efflux pumps, conferring resistance to macrolides and fluoroquinolones, and *Alm*G, encoding resistance to polymyxins, was observed across all *V. cholerae* isolates in this study, indicative of broad-spectrum resistance mechanisms. The *emr*D multidrug transporter efflux pumps were exclusively found in the isolates from clinical sources (MMRF1099) and Vembanad lake (MMRF1896). Furthermore, the clinical isolate exhibited resistance genes *sul*II and *aph*6, *aph*3d, conferring resistance to sulfamethoxazole and aminoglycosides, respectively. While *V. cholerae* isolates showcased diverse resistance genes, *V. alginolyticus* isolates exhibited a more consistent resistance profile characterised by intrinsic resistance genes $bla_{CARB}$, *tet*34, and *tet*35, conferring resistance to beta-lactams and tetracycline. Remarkably, a subset of isolates, namely MMRF2244, MMRF2258, and MMRF2260, exhibited the additional presence of CRP efflux pumps. Conversely, analysis of *V. vulnificus* isolates revealed a more streamlined resistance profile, characterised primarily by the sole presence of the putative *tet*34 gene. These findings underscore a distinct resistance mechanism employed by *Vibrio* spp., potentially reflecting species-specific adaptations to antimicrobial challenges (Fig. 3D).

After comparing the antimicrobial resistance profiles of *V. cholerae* isolates we collected in this study with those from other isolates referred for SNP phylogeny, it was found that quinolone resistance is predominantly reported in nonO1/nonO139 strains originating from clinical settings. Notably, quinolone resistance is most frequently documented in Asia compared to other geographical regions (Fig. 2A). In comparing the antimicrobial resistance patterns of *V. vulnificus*, the study revealed that the *tet*34 gene, which encodes tetracycline resistance, was uniformly distributed in all isolates from food, the environment, and clinical sources, suggesting that this trait could be intrinsic to this group of bacteria (Fig. 2C). In contrast, *V. algniolyticus* strains were genetically diverse, and formed three distinct clades, with 4 out of 5 isolates carrying the multidrug efflux pump CRP, conferring resistance to fluoroquinolones and macrolides. Interestingly, CRP was not uniformly found in *V. alginolyticus* strains, despite being a widely distributed global regulator that represses MdtEF multidrug efflux pump expression in Gram-negative bacteria (Fig. 2D). Screening for mobile genetic elements within the genomes of the isolates obtained in this study did not reveal the presence of any plasmid sequences. However, the analysis identified insertion sequences in all isolates except *V. alginolyticus* MMRF2258 and complete phage sequences were detected only in *V. alginolyticus* genomes, and none of the mobile genetic elements detected were found to be associated with antimicrobial resistance or virulence. A comprehensive summary of the identified phage and insertion sequences can be found in Table 3.

### 3.4. Characterisation of virulent factors in Vibrio isolates

An overview of the virulent factors identified in the isolates is provided in Fig. 3. Screening for genes encoding virulence revealed that *V. cholerae* isolates carried a wide range of virulent factors compared to the marine isolates of *V. vulnificus* and *V. alginolyticus* used in this study. All *V. cholerae* isolates carried genes of multifunctional autoprocessing RTX toxin machinery and virulence-associated secretion (vas) genes of T6SS, which comprises *vgr*G, *vip*A, *vip*B, *clp*B, *icm*F, *vas*A, *vas*B, *vas*C, *vas*D, *vas*E, *vas*F, *vas*G, *vas*H, *vas*I, *vas*J, VCA0109 and VCA0122. Clinical isolate of *V. cholerae* (MMRF1099) carried virulence factors, including *ctx* (cholera toxin), *zot* (zonula occludens toxin), *ace* (accessory cholera enterotoxin), TCP (toxin-coregulated pilus) and ACF (accessory colonization factor) which were absent in the freshwater and marine isolates. Interestingly, we couldn't identify type VI secretion system *hcp*-2 in the genome of clinical isolate (MMRF 1099), compared to all other isolates of *V. cholerae* reported in this study. The *V. alginolyticus* strains carried genes for T3SS and harboured the Thermolabile hemolysin-encoding *tlh*

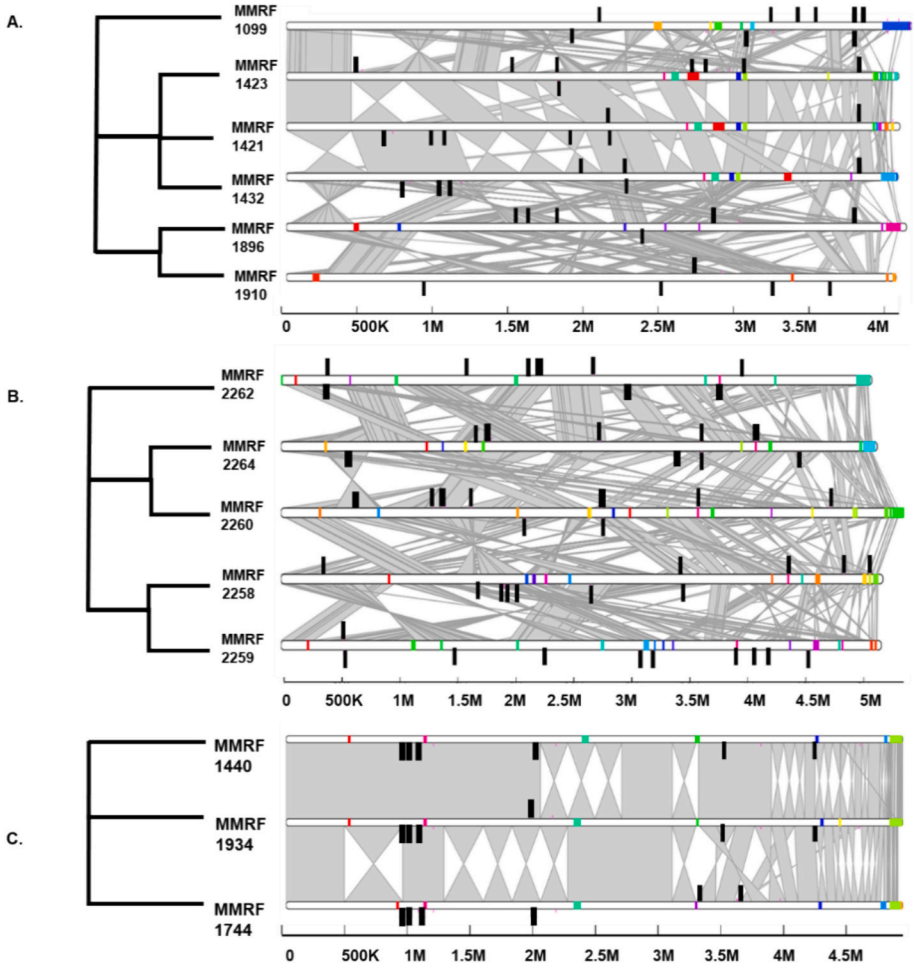


**Fig. 1.** Comparison of genomic islands between, A. *V. cholerae*, B. *V. algniolyticus* and C. *V. vulnificus* isolates collected in this study. Grey shaded regions indicate the regions of homology, black coloured blocks indicate the location of AMR genes in the isolate whereas other coloured blocks represent genomic islands observed across each strain.

gene. *V. vulnificus* isolates exhibited a consistent virulence pattern, carrying only a limited number of genes such as *omp*U, *Ilp*A (Immunogenic lipoprotein A), and *rtx*.

### 3.5. In-silico MLST analysis and pathogen prediction

The MLST analysis revealed the presence of novel alleles at the metE locus in all environmental isolates, whereas the clinical isolate exhibited sequence type (ST69), corresponding to the seventh pandemic clone. Additionally, novel alleles were detected at loci *mdh*, *pnt*A, *lys*A, *dtd*S, *tna*A, *pyr*C, and *glp* in the *V. vulnificus* isolates examined, posing challenges in accurately determining their sequence types. This study did not conduct MLST analysis for *V. alginolyticus* isolates because no suitable scheme was available in PubMLST. All *Vibrio* isolates harboured genes belonging to pathogenic families and were identified as human pathogens according to the PathogenFinder database few of which include flagellar hook-associated proteins (FliD), putative outer membrane protein and haemolysin.

## 4. Discussion

The adaptability of *Vibrio* spp. to coastal environments has emerged as a global public health concern, driven by rising cases of severe wound, skin, and gastrointestinal infections linked to contaminated waters. The O1 El Tor strain of *V. cholerae* (MMRF 1099) identified in this study was isolated from the stool of a cholera patient who had travelled from West Bengal to Kerala (Krishna et al., 2020). Upon comparison, the genome of MMRF 1099 was found to be identical to the genomes of O1 serotypes available in the NCBI database, most of which originated from Asia, with a similar trend of clonal expansion observed for this serotype in Africa, Europe, and Australia. The El Tor biotype, responsible for the ongoing 7th pandemic, was initially reported from Indonesia in 1961 and has displaced the classic biotype globally (Hu et al., 2016). Several studies have accounted for the circulation of El Tor, the Haitian variant and several atypical El Tor variants of *V. cholerae* in the Indian subcontinent (Narendrakumar et al., 2020). Human mobility within the Indian subcontinent has markedly increased over the past decade, primarily driven by industrialisation and enhanced transportation networks. This phenomenon has facilitated the dissemination of cholera-infected individuals across various regions, raising significant concerns regarding genetic mixing among *V. cholerae* strains. In Kerala, there has been a notable rise in cholera incidence, particularly among migratory workers, despite the presence of robust public health systems. Between 2010 and 2015, three districts reported cholera in at least three out of five consecutive years (Ali et al., 2017). The situation intensified in July 2024, when 22 suspected cases, including two confirmed instances, were identified in the Thiruvananthapuram district. The concurrent isolation of an El Tor variant from coastal waters underscores the role of marine environments as reservoirs for pathogenic *V. cholerae* (Ayyappan et al., 2024) Comparable patterns have been observed in other global coastal regions, including the Bay of Bengal and Latin America, where both toxigenic and non-toxigenic strains persist in estuarine and marine

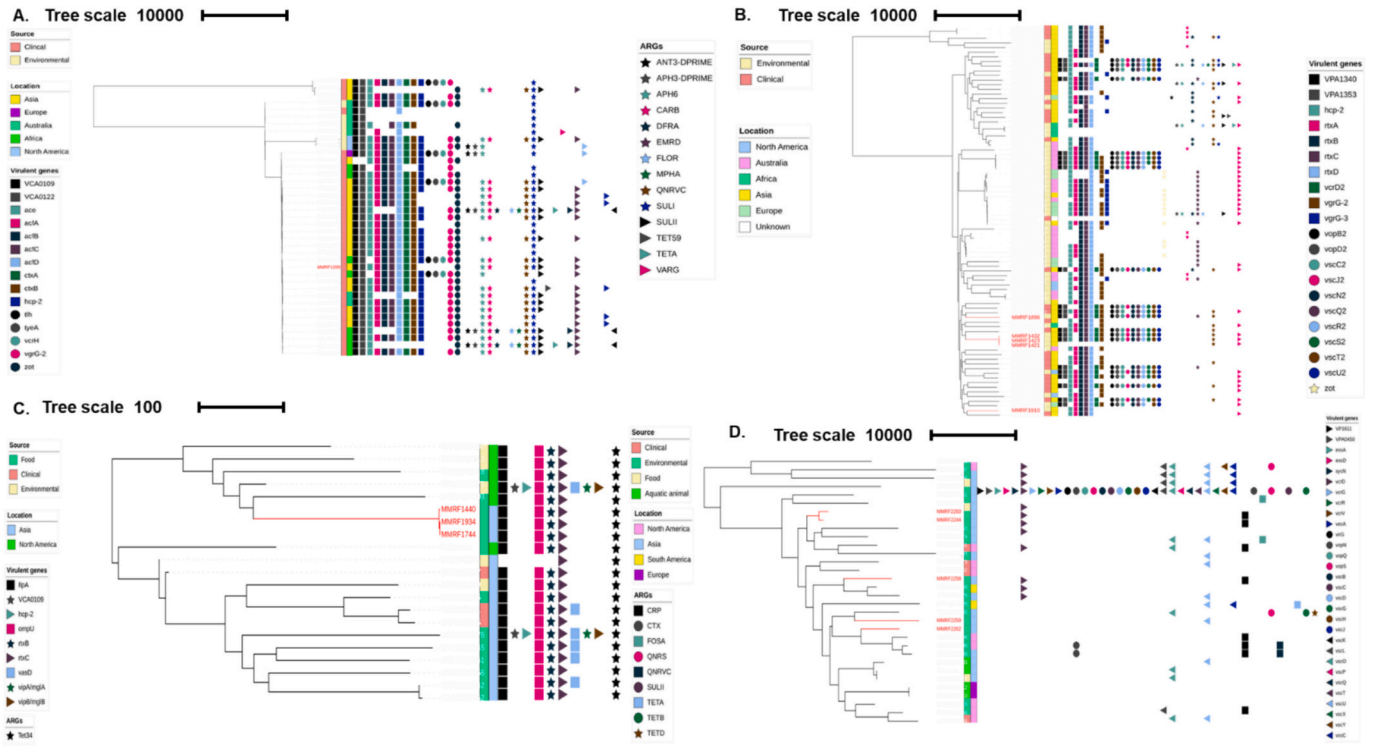


**Fig. 2.** SNP-based phylogeny with a comparison of virulent and antimicrobial resistant genes between isolates from this study (red lines) with reference isolates collected from various locations and sources A. *V. cholerae* O1 serotype B. *V. cholerae* non-O1/non-O139 C. *V. vulnificus* D. *V. alginolyticus.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


**Fig. 3.** Overview of virulent genes detected in the genome of A. *V. cholerae* (yellow) B. *V. alginolyticus* (green) C. *V. vulnificus* (red) described in this study. D. Overview of antimicrobial-resistant genes (purple) identified in the *Vibrio* spp. isolated from this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

environments (Huq et al., 2005; Lipp et al., 2002).

Environmental isolates of *V. cholerae* were predominantly non-O1/non-O139 serotypes and formed distinct clusters. Isolates from the Arabian Sea and Bay of Bengal coasts were grouped, while those from groundwater and Vembanad Lake formed separate clusters. All strains were genetically similar to non-O1/non-O139 serotypes found in clinical samples of Asian origin. These serotypes are a growing global concern due to their transmission through contaminated food and recreational contact with coastal waters (Bhandari et al., 2023; Schmidt et al., 2023; Tang et al., 2023). There were multiple cases of mortalities reported in China due to infection caused by non-O1/non-O139 serotypes of *V. cholerae* transmitted through the consumption of contaminated sea-food and recreational activities (Hwang et al., 2021); (Zhao et al., 2022). It is evident from the literature that the infections caused by O1/O139

**Table 3**
An overview of mobile genetic elements and phages identified in the genomes of *Vibrio* isolates collected in this study.

| Name of the isolate | Mobile genetic elements | Phage sequences |
|---|---|---|
| *V. cholerae* MMRF 1432 | IS630, ISAS1, IS481, IS4, IS5, IS200/IS605 | – |
| *V. cholerae* MMRF 1896 | IS3 | – |
| *V. cholerae* MMRF 1099 | ISAS1, IS21, IS481, IS91, IS256, IS5 | – |
| *V. cholerae* MMRF1423 | IS630, ISAS1, IS5, IS200/ IS605 | – |
| *V. cholerae* MMRF 1421 | IS630, IS481, ISAS1, IS5, IS200/IS605 | – |
| *V. cholerae* MMRF 1910 | IS5, IS200/ IS605 | – |
| *V. vulnificus* MMRF 1440 | ISNCY, IS5, IS4, IS30 | – |
| *V. vulnificus* MMRF 1744 | ISNCY, IS5, IS4, IS30 | – |
| *V. vulnificus* MMRF1934 | ISNCY, IS5, IS4, IS30 | – |
| *V. alginolyticus* MMRF2244 | IS3, IS481, IS200/IS605 | PHAGE_Vibrio_VFJ, PHAGE_Escher_vB_EcoM_ECOO78, PHAGE_Vibrio_VEJphi |
| *V. alginolyticus* MMRF2258 | – | PHAGE_Entero_Arya |
| *V. alginolyticus* MMRF2259 | IS481, IS200/ IS605 | – |
| *V. alginolyticus* MMRF2260 | IS3, IS481, IS110 | PHAGE_Entero_SfV, PHAGE_Vibrio_K139, PHAGE_Entero_HK225, PHAGE_Yersin_PY54 |
| *V. alginolyticus* MMRF2262 | IS200/IS605 | – |

and non-O1/non-O139 serotypes may not follow similar clinical manifestations in humans due to their genetic diversity (Gupta et al., 2022). Most of the clinical manifestations of infections caused by O1/O139 serotypes are mediated through the action of cholerae toxin coded by the *ctx* gene (Faruque et al., 1998), while non-O1/non-O139 possess diverse virulence mechanisms which are either coded by T6SS, T3SS, CT or their combinations (Igere et al., 2022). The multifunctional auto-processing RTX toxin machinery and virulence-associated secretion genes of Type 6 Secretion System (T6SS) genes (*vgr*G, *vip*A, *vip*B, *clp*B, *icm*F, *vas*A, *vas*B, *vas*C, *vas*D, *vas*E, *vas*F, *vas*G, *vas*H, *vas*I, *vas*J, VCA0109 and VCA0122) were found commonly in both clinical and environmental isolates of *V. cholerae* in the current study. Among these genes, *vgr*G aids the penetration of the pathogen through the intestinal membranes of host organisms (Brooks et al., 2013), while *vip*A, *vip*B, VCA0109, and vas encode structural components that facilitate the release of toxin Hcp (Zheng et al., 2011). However, one of the significant genes (*hcp*) found in other clinical isolates responsible for the action of hemolysin coregulated protein was not observed in the genome of the clinical isolate, *V. cholerae* (MMRF1099) and the isolate from Vembanad Lake (MMR1896). At the same time, it was also present in the remaining environmental isolates from the Arabian Sea and the Bay of Bengal (MMRF1421, MMRF1423, MMRF1432, and MMRF1910). Therefore, the pathogenesis of MMRF1099 could be mediated through the expression of enterotoxin CT, *zot*, and *ace*. CT disrupts active ionic transport in the small intestine by binding to the GM1 ganglioside receptor of ileal cells, *zot* induces structural changes to tight junctions of epithelia (Uzzau et al., 1999) and *ace* results in fluid secretion in the ileum (Trucksis et al., 1993). In strains lacking *tcp* and CT, T3SS mediates the adhesion of bacterial cells to intestinal epithelia and secretion of hemolysin (*Hly*A) and RTX toxin, leading to cell damage and cell death of host cells (Ramamurthy et al., 2020). It is worth noting that *V. cholerae* non-O1/non-O139 strains in this study carried genes encoding the T3SS, a characteristic of *V. parahaemolyticus*, whereas this virulence factor was absent in the clinical isolate. This suggests a possible evolutionary divergence between non-O1/non-O139 and O1/O139 strains of *V. cholerae*. Moreover, variation in genomic islands within strains may indicate niche-specific adaptation of these bacteria in their environment. The differences in the expression of virulence and the transmission routes of pathogens also highlight the need to revise the WaSH (Water, Sanitation, and Hygiene) protocol, as it currently overlooks the transmission of diseases from coastal waters to humans. The revised strategies may consider risk maps of microbially polluted water bodies, which can be generated using in situ, citizen science and earth observation-based surveillance data.

Apart from *V. cholerae*, we also studied the genomes of *V. vulnificus* and *V. alginolyticus* isolated from coastal waters. Wound infections and fatalities caused by *V. vulnificus* of marine origin are on the rise, and their link with indicators of climate change, such as global warming, is reported in the US and Europe (Paz et al., 2007; Urquhart et al., 2014) while their incidence is not well studied in the Indian subcontinent. The nearest phylogenetic neighbours of *V. vulnificus* in Indian coastal areas (MMRF 1440, 1744 and 1934) were reported from food sources in North America. The *V. vulnificus* isolates in the current study possessed virulence genes, *rtx*B, *rtx*C, *omp*U, and *llp*, which were observed in all isolates compared. The presence of *rtx* and *omp*U genes confirms that MMRF1440, 1744, and 1934 are biotype 2, which can cause human infections (Jones and Oliver, 2009). Massive wound infections in humans caused by *V. vulnificus* were reported from the Mediterranean basin, North America, and Europe (Paz et al., 2007; Urquhart et al., 2014). The *V. alginolyticus* isolates in this study formed distinct clusters with clinical and environmental strains from Asia and the Americas, while none harboured the *tdh* or *trh* genes, considered putative virulence markers in *V. alginolyticus* (Hernández-Robles et al., 2016). However, these isolates had the components of the T3SS1 system (*tye*A), T3SS translocated genes (*vop*D, *vop*B, and *vcr*H), *vsc*I and *vsc*F, and hemolysin, *tlh* (Supplementary Table 3). The expression of these genes has the potential to induce pathogenicity by facilitating the release of virulence-effector proteins into host cells, thereby triggering apoptosis and necrosis within those cells (Wong et al., 2012). Furthermore, infections have been documented among individuals who engage with coastal waters during the warmer seasons (Sganga et al., 2009; Zhou et al., 2021). On the other hand, some of these isolates are proposed as potential probiotics for marine animals, indicating their ecological significance (Xie et al., 2005).

Our previous studies showed that more than 65 % of *Vibrio* species isolated from the coastal waters of India acquired resistance to ampicillin, cefalexin, cefepime, cefoxitin, erythromycin, gentamicin, meropenem, moxifloxacin, and trimethoprim (Abdulaziz et al., 2023). This resistance pattern is supported by the presence of the *alm*G (polymyxin), *crp* (penam, fluoroquinolone, macrolide), and *qnr*VC (fluoroquinolone) genes in the genome of *V. cholerae* in the current study. The genome of *V. cholerae* isolated from the coastal waters of the Arabian Sea and the Bay of Bengal shared a similar pattern of antibiotic resistance genes, different from those isolated from other environmental (Vembanad lake and groundwater) and clinical sources. The results of the disc diffusion assay suggested that the expression of resistance genes is conditional and varies between isolates. The isolates of *V. cholerae* obtained from groundwater (MMRF 1910) and Vembanad Lake (MMRF 1910) expressed a low resistance level to multiple antibiotics, as evidenced by a MAR index of less than 0.25. In contrast, the *V. cholerae* strains derived from other sources, including clinical settings and the coastal areas of the Arabian Sea and the Bay of Bengal, exhibited a MAR index of 0.5 or greater. Quinolone resistance, exclusively found in marine *V. cholerae*, could indicate that marine ecosystems are a potential reservoir of residual antibiotics that have selected for resistance. Coastal waters are prone to contamination from multiple sources, including terrestrial and marine ecosystems, which might help vibrios acquire diverse resistance mechanisms towards antibiotics. Despite *V. cholerae*'s ability to

efficiently acquire extracellular DNA (Matthey and Blokesch, 2016), integrative and conjugative elements carrying antimicrobial resistance determinants have not been found in environmental *V. cholerae* strains. Additionally, all sequenced clinical and environmental strains tested negative for class I integron, which is consistent with previous studies suggesting limited spreading of antibiotic resistance genes through mobile genetic elements in non-epidemic regions (Bier et al., 2015) (Baron et al., 2017; Ceccarelli et al., 2016).

The presence of genes (*qnr*VC) conferring resistance to the latest generation of antibiotics, such as fluoroquinolones, has been reported among clinical isolates of *V. cholerae* from the Indian subcontinent (Kumar et al., 2017). The current study confirmed the presence of these genes in isolates from coastal environments, namely MMRF1432, MMRF1423, MMRF1896, MMRF1421. The presence of *V. cholerae* carrying genes that confer resistance to multiple antibiotics, including β-lactams and macrolides, has been reported in coastal waters around the world, leading to cases of sepsis in humans (Bier et al., 2015; Jeamsripong et al., 2022; Rashed et al., 2017; Xu et al., 2023; Zhang et al., 2023). The antimicrobial resistance pattern often mirrors antibiotic usage in terrestrial environments (Larsson et al., 2023). This correlation extends to the resistance patterns observed in *V. cholerae* across different coastal environments. For instance, a study by (Ceccarelli et al., 2016) reported that *V. cholerae* non-O1/non-O139 isolates demonstrated resistance to ampicillin and streptomycin, findings that align with another study from German coastal waters by (Bier et al., 2015). Resistance to newer generations of antibiotics, such as fluoroquinolone, indicates the challenges in treating individuals infected with resistant forms of *V. cholerae* non-O1/non-O139. Since we employed short-read sequencing, our analysis may not capture the entire genetic repertoire of the isolates, especially those linked to plasmids, which may have gone missing from our reconstructed genome (Sundquist et al., 2007).

India has historically been a major epicentre for infections caused by *V. cholerae*, yet many cases likely go undiagnosed or misclassified due to limited molecular diagnostics. Infections from other *Vibrio* spp. are also rising, including *V. vulnificus*-related wound infections, septicaemia from non-O1/non-O139 *V. cholerae*, and foodborne illness linked to *V. parahaemolyticus*. Since these pathogens are present in aquatic reservoirs such as water, plankton, and seafood, there is an urgent need to map exposure risks through molecular surveillance. Engaging citizen scientists in seasonal water sampling, coupled with qPCR-based screening in authorised water laboratories, could support early detection. Such grassroots efforts, aligned with strengthened molecular surveillance, may support outbreak preparedness, guide interventions, and advance insights into antimicrobial resistance, ultimately informing public health strategies and policy frameworks.

## CRediT authorship contribution statement

**P.S. Seethalakshmi:** Writing – original draft, Software, Data curation, Formal analysis. **Abdulaziz Anas:** Writing – review & editing, Supervision, Investigation, Conceptualization. **K. Devika Raj:** Visualization, Validation, Data curation. **C. Jasmin:** Supervision, Investigation. **Nandini Menon:** Project administration, Writing - review & editing. **Grinson George:** Project administration, Writing - review & editing. **Shubha Sathyendranath:** Project administration, Funding acquisition, Writing - review & editing.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.118496.

## Data availability

The whole-genome sequence data generated for this study were deposited GenBank. Supplementary file, Supplementary Table 1.

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