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Preliminary characterization of *Vibrio cholerae* strains isolated from seafood samples marketed in Portugal

Teresa Lopes [a], David Lopes [b], Isabel Bastos Moura [a], Isabel Sousa [c], João Rodrigues [d], Camila Fernandes [e], Maria João Barreira [f], Carla Maia [f], Cristina Belo Correia [f], Cristina Pintado [b,g], Margarida Saraiva [a], Rita Batista [f,*]

[a] *National Institute of Health Doctor Ricardo Jorge, Department of Food and Nutrition, Rua Alexandre Herculano 321, 4000-055, Oporto, Portugal*
[b] *School of Agriculture, Polytechnic University of Castelo Branco, 6001-909, Castelo Branco, Portugal*
[c] *National Institute of Health Doctor Ricardo Jorge, Culture Media Preparation Laboratory, Rua Alexandre Herculano 321, 4000-055, Oporto, Portugal*
[d] *National Institute of Health Doctor Ricardo Jorge, Department of Infectious Diseases, Avenida Padre Cruz, 1649-016, Lisbon, Portugal*
[e] *National Institute of Health Doctor Ricardo Jorge, Human Genetics Department, Avenida Padre Cruz, 1649-016, Lisbon, Portugal*
[f] *National Institute of Health Doctor Ricardo Jorge, Department of Food and Nutrition, Avenida Padre Cruz, 1649-016, Lisbon, Portugal*
[g] *Research Centre for Natural Resources, Environment and Society (CERNAS), Polytechnic University of Castelo Branco, Av. Pedro Álvares Cabral, 12, 6000-084, Castelo Branco, Portugal*

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ABSTRACT

Vibrio cholerae, a natural inhabitant of aquatic ecosystems, has been related with gastrointestinal infections, particularly those associated with seafood consumption. This preliminary study aimed to evaluate the presence and characteristics of *Vibrio cholerae,* in seafood marketed in Portugal, given its potential role as a foodborne pathogen. Thus, the occurrence of *Vibrio cholerae* in 129 seafood raw samples (105 of shrimp and 24 of oysters), marketed in Portugal, was assessed. Isolates' characterization regarding the presence of antimicrobial resistance (AMR) genes and of pathogenicity-specific genetic traits was attained by whole-genome sequencing (WGS). Core-genome Multi Locus Sequence Typing (cgMLST) analyses to evaluate the genetic relatedness among the isolates, and with other *V. cholerae* strains isolated in the world, as well as phenotypic AMR (performed by disc diffusion), were also attained. Overall, 43/129 (33.3 %) of the samples tested positive for *V. cholerae* (41/105 (39.1 %) of the shrimp and 2/24 (8.3 %) of the oysters' samples). WGS analyses classified the studied strains as non-O1/non-O139 *Vibrio cholerae* (NOVC), lacking the main cholerae virulence factors encoded by the CTX phage. However, they carry diverse virulence factors similar to those found in O1 and O139 strains and/or in NOVC clinical strains. Furthermore, eight strains were classified as multidrug-resistant (MDR). The cg-MLST analyses revealed six genetic clusters among the 43 isolates (three identified sequence types - ST829, ST833, ST1085). Although it was not possible to find a close genetic relatedness between the studied *V. cholerae* strains and other deposited in PuBMLST database, a high genetic proximity among some strains isolated in different countries and from different sources (environmental and human) was observed, reinforcing the importance of a One Health approach. Assessing occurrence, pathogenic potential and genetic relatedness of *Vibrio cholerae* strains in the Portuguese food supply chain, this study contributes to understand their public health significance and supports a One Health approach to prevent foodborne outbreaks, contributing to food safety.

1. Introduction

It is now well accepted that *Vibrio cholerae,* the causative agent of the disease cholera, and other non-cholera *Vibrio* spp., are acquired from various aquatic environments including marine, estuarine and freshwater systems (Mavian et al., 2020; Reidl and Klose, 2002).

* Corresponding author.
E-mail addresses: teresa.lopes@insa.min-saude.pt (T. Lopes), davidlopes978@hotmail.com (D. Lopes), isabel.moura@insa.min-saude.pt (I.B. Moura), rosa.sousa@insa.min-saude.pt (I. Sousa), joao.rodrigues@insa.min-saude.pt (J. Rodrigues), camila.fernandes@insa.min-saude.pt (C. Fernandes), m.joao.barreira@insa.min-saude.pt (M.J. Barreira), carla.maia@insa.min-saude.pt (C. Maia), cristina.belo@insa.min-saude.pt (C.B. Correia), cpintado@ipcb.pt (C. Pintado), margarida.saraiva@insa.min-saude.pt (M. Saraiva), rita.batista@insa.min-saude.pt (R. Batista).

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Environmental factors such as climate change and water pollution can increase the prevalence of pathogenic *Vibrio* spp. in these environments (Le Roux et al., 2015, Trinanes and Martinez-Urtaza, 2021). Fish, shrimp and other aquatic animals, can become infected through exposure to contaminated water. Moreover, shrimp and mollusc bivalve shells are surfaces where *V. cholerae* can form biofilms, which protect bacteria from environmental stress and enhance their ability to infect. These shells also contain chitin, a natural polymer that *V. cholerae* can use as nutrient source (Nahar et al., 2012). The association of *V. cholerae* with aquatic organisms like copepods (small crustaceans) and chironomids (non-biting midges), organisms that provide nutrients and protection, facilitates the bacteria survival but also its dissemination, since these organisms are part of other species' diet, namely shrimp, fish and waterbird species. In fact, migratory movements of fish and waterbirds provide a possible vehicle of local and/or intercontinental transport of *V. cholerae* (Laviad-Shitrit et al., 2019).

The spread of *V. cholerae* to humans occurs typically through the consumption of contaminated water or raw or undercooked seafood, or through the contact with contaminated work surfaces. When infected, humans disseminate the bacteria though the faecal-oral route.

Although a portion of shrimp consumed in Europe is imported from some countries where cholera is endemic, it is important to emphasize that the risk of cholera transmission through imported shrimp is considered low, with all reported European cholera cases having a travel history to cholera-affected areas (https://www.ecdc.europa.eu/en/all-topics-z/cholera/surveillance-and-disease-data/cholera-monthly). However, toxicogenic O1 and O139 *V. cholerae* strains were already isolated from seafood marketed in Malaysia (Chen et al., 2004; Elhadi et al., 2004), a country from where European Union imports seafood (https://wits.worldbank.org/trade/comtrade/en/country/EUN/year/2023/tradeflow/Imports/partner/ALL/product/030613).

Cholera toxin (CTX) and toxin-coregulated pilus (TCP) are two major virulence factors produced by epidemic *V. cholerae* strains of serotypes O1 and O139 (Montero et al., 2023). Nevertheless, some non-O1/O139 strains that do not produce cholera toxin and toxin-coregulated pilus have been reported to cause sporadic diarrhoea and gastroenteritis in humans, indicating the existence of other virulence factors (Dalsgaard et al., 1995; Schirmeister et al., 2014; Trubiano et al., 2014)

Non-O1/non-O139 *V. cholerae* (NOVC) are strains of highest relevance for public health in the European Union (EU) through seafood consumption (EFSA Panel on Biological Hazards (BIOHAZ), 2024). In fact, in the last five years 53 Rapid Alert System for Food and Feed (RASFF) notifications were presented regarding the detection of *Vibrio cholerae* in seafood in Europe (RASFF Window – search). The European Food Safety Authority (EFSA) acknowledge the existence of gaps related to *Vibrio* spp. in seafood and aquatic environments such as the absence of robust data on the proportion of *Vibrio* spp. isolates from seafood marketed in the EU displaying specific genetic traits associated with pathogenicity.

In this preliminary investigation we aimed to assess the occurrence of *V. cholerae* in seafood samples (shrimp and oysters) marketed in Portugal, and to characterize the isolated *V. cholerae* strains, namely its potential virulence, antimicrobial resistance and its genetic relatedness with other *V. cholerae* strains isolated in other studies from different places in the world.

2. Material and methods

2.1. Sampling

One hundred and twenty-nine (129) individual seafood raw samples (105 of frozen shrimp and 24 of fresh oysters), marketed in Portugal were tested for the presence of *V. cholerae,* from May 2023 to June 2024. Samples were stored at frozen and refrigeration temperatures (-18 °C and 1 °C to 5 °C respectively) after collecting/ purchasing until processing (within 24 h for the fresh ones), and analyzed during their assigned shelf-life period.

2.2. Vibrio cholerae detection and isolation

Isolation of *V. cholerae* was performed according to ISO 21872–1:2017/Amd1:2023. Each sample was homogenised (30–100 g) and then a test portion of 25 g of the food sample was used to prepare a 1:10 initial suspension in Alkaline Saline Peptone Water (ASPW) (Oxoid, Basingstoke, Hampshire, UK). After incubation of the initial suspension at 37 °C ± 1 °C for frozen shrimps and 41,5 °C ± 1 °C for fresh oysters during 6 h ± 1 h, 1 ml of the culture was transferred into a tube containing 10 ml of ASPW (secondary enrichment) and incubated at 41, 5 °C ± 1 °C for 18 h ± 1 h. PCR was used to screen for the presence of target bacterium through a specific genetic marker used to identify *Vibrio cholerae* (*prVC* gene) and toxin-encoding genes (*ctxA e ctxB*) in secondary enrichment broths, following incubation (see Table 1, for PCR details).

When the target sequence, specific of *V. cholerae,* was present, thiosulfate, citrate, bile and sucrose (TCBS) agar plates (Biokar Diagnostics, Pantin, France) and *Vibrio* Chromogenic Agar plates (Condalab, Torrejón de Ardoz, Madrid, Spain) were used for plating-out and incubated for 24 h ± 3 h at 37 °C ± 1 °C. Colonies of presumptive *V. cholerae* (slightly flattened, with opaque centers and translucent peripheries, yellow colonies on TCBS agar, and pink to red colonies on Chromogenic agar) were isolated in Saline Nutrient Agar (SNA- homemade) or Columbia Agar with 5 % Sheep Blood (bioMérieux, Marcyl l'Etoile, France). The identification of characteristic strains was performed by conventional PCR, following ISO_21872–1:2017/Amd1:2023 protocol (detection of *prVC* - Table 1) and MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) approaches (Autof MS1000, Autobio Diagnostics, Zhengzhou, China). All positive isolates were stored in Tryptone Soy Broth (TSB; Biokar Diagnostics) with 20 % glycerol, at −80 °C.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed by disc diffusion in thirty-nine (39) *V. cholerae* isolates, for six antimicrobials: Ampicillin (AMP), Ceftazidime (CZD), Ciprofloxacin (CIP), Chloramphenicol (CHL), Meropenem (MEM) and Piperacillin-tazobactam (PTZ). Four of the 43 strains were not tested since they became not culturable after being cryopreserved. For the antimicrobials Ampicillin and Chloramphenicol the interpretative criteria specific for *Vibrio spp*., including *V. cholerae* described in CLSI document M45 3rd edition (CLSI, 2015) was followed. For the other tested antimicrobials, the assay was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (The European Committee on Antimicrobial Susceptibility Testing, 2024), and EUCAST epidemiological cut-off values (ECOFFs) were used for the results interpretation. Isolates were classified as multidrug-resistant (MDR) when showing resistance to three or more antimicrobial classes (Magiorakos et al., 2012).

Data from the *Vibrio cholerae* O1 El Tor strain 2010EL-1786 (ATCC BAA-2163) susceptibility report was included for perspective (BAA-2163 %20Antibiotic%20Susceptibility%20Report.pdf).

2.4. Whole-genome sequencing (WGS), in silico typing and screening of virulence/AMR genes

ISOLATE II Genomic DNA Kit (Bioline, London, England, UK) was used for the extraction of genomic DNA from fresh cultures of all *V. cholerae* isolates. DNA quantification was performed in the Qubit fluorometer (Invitrogen, Waltham, MA. USA) with the 1x dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Indexed DNA libraries were then prepared using the Nextera XT library preparation kit (Illumina, San Diego,

Table 1
PCR details for the detection of *prVC, ctxA and ctxB Vibrio cholerae* genetic elements.

Target/ amplicon size	Primer sequences	PCR cycling parameters	References
prVC/ 295–310 bp	*prVC*(FW): TTA AGC STT TTC RCT GAG AAT G *prVC*(REV): AGT CAC TTA ACC ATA CAA CCC G	94 °C, 2 min (1 cycle); 94 °C, 1 min; 50 °C, 1 min, 72 °C, 2 min (30 cycles); 72 °C, 10 min (1 cycle)	Chun et al., 1999; ISO_21872-1:2017/Amd1:2023
ctxA/ 564 bp	CTX2(FW): CGG GCA GAT TCT AGA CCT CCT G CTX3(REV): CGA TGA TCT TGG AGC ATT CCC AC	95 °C, 5 min (1 cycle); 95 °C, 1 min; 60 °C, 1 min, 72 °C, 1 min (25 cycles); 72 °C, 10 min (1 cycle)	Fields et al., 1992
ctxB/ 460 bp	CTX7(FW): GGT TGC TTC TCA TCA TCG AAC CAC CTX9B(REV): GAT ACA CAT AAT AGA ATT AAG GAT	95 °C, 5 min (1 cycle); 95 °C, 1 min; 55 °C, 1 min, 72 °C, 1 min (30 cycles); 72 °C, 10 min (1 cycle)	Olsvik et al., 1993

prVC- *Vibrio cholerae* 16–23S rRNA intergenic spacer region, ***ctxA***- A subunit of cholera toxin; ***ctxB***- B subunit of cholera toxin; **bp**- base pairs

CA, USA). Assessment of DNA library profile and concentration was determined using the Fragment Analyser System (Agilent Technologies). Sequencing runs were performed on either a MiSeq, a NextSeq 550 or a NextSeq 2000 instrument (Illumina) using 2 × 150 bp reads. Raw sequencing reads were automatically demultiplexed on-instrument following the end of each run.

V. cholerae sequencing reads were treated on Galaxy Aries platform (Galaxy (iss.it)). FastQ positional and quality trimming (Galaxy Version 0.0.1) (Cuccuru et al., 2014) was used for trimming, FastQC Read quality Reports (Galaxy Version 0.72 +galaxy1) (Andrews, 2010) for quality check of sequencing reads, SPAdes (Galaxy Version 3.14.1 +galaxy1) (Bankevich et al., 2012) for genome assembly, Filter SPAdes repeats (Galaxy Version 1.0.1) (Iskander, 2004) for removing short and repeated contigs/scaffolds, Quast (Galaxy Version 5.0.2 +galaxy1) (Mikheenko et al. 2016a, 2016b, 2018; Gurevich et al. 2013) for checking the quality of the genome assemblies and Kraken2 (Galaxy Version 2.1.3 +galaxy1) (Wood and Salzberg, 2014) for species confirmation/ contamination screening.

Vibrio cholerae assemblies were submitted to PubMLST platform (https://pubmlst.org/) where the sequence types (ST) were determined.

Screening of Antimicrobial resistant genes was performed with Resistant Gene Identifier (RGI) (Galaxy Version 4.2.2) (Jia et al. 2017) on Galaxy Aries platform (Galaxy (iss.it)). *Vibrio cholerae* O1 str 2010EL-1786 (ATCC BAA-2163) assembly (NCBI RefSeq assembly: GCF_000166455.1) was included in this analysis for perspective.

Mapping of *V. cholerae* strains virulence genes was performed with Nucleotide BLAST (BLASTn), on NCBI platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using *V. cholerae* O1 biovar El Tor str. N16961 as reference (Heidelberg et al., 2000). In this analysis the presence/absence of 177 virulence genes was determined. Furthermore, the presence/absence of *wbeO1* (GenBank: KC152957) and *wbfO139* (GenBank: AB012956) gene clusters encoding the somatic O antigens, specific of O1 (Xu et al., 2013) and O139 (Yamasaki et al., 1999) serotypes, respectively, of the *stn* gene (GenBank: M85198), encoding the heat-stable enterotoxin (Ogawa et al., 1990) and of the *chxA* gene, encoding Cholix toxin (GenBank: KY595959.1) were also checked by BLASTn on NCBI platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (list of analyzed genes and accession IDs on Supplementary Table 1). The presence of a gene/gene cluster was defined when the coverage and gene identity were higher than 70 % and 75 %, respectively. For those strains where the *nanh* gene, encoding for neuraminidase, was detected, an alignment with *Vibrio* pathogenicity island-2 (VPI-2) of strain Amazonia isolate 3509 (GenBank: EU272902.1) was performed, in order to check for the detection of a complete VPI-2. The detection of the type three secretion system (T3SS) was also checked by BLASTn using as query sequence the R-20000 T3SS gene cluster (GenBank: MH494081.1). The presence of a complete VPI-2 and/or T3SS was considered when the coverage and identity were higher than 85 % and 95 %, respectively.

Sequencing reads were deposited on the European Nucleotide Archive (ENA) under de bioproject PRJEB85234. Accession numbers as well as information about genome assemblies' quality assessment for each isolate are listed in Table 2.

2.5. Phylogenetic and clustering analysis of Vibrio cholerae isolates

A phylogenetic tree of the 43 whole-genome sequenced *Vibrio cholerae* isolates was performed on PUBMLST with iTOL (v6) (Letunic and Bork, 2021) (https://itol.embl.de/) and based on a 2457 loci schema (all loci). This tree was rooted on *V. cholerae* O1 biovar El Tor str. N16961 (AE003852.1) and a sequence of *Vibrio mimicus* strain NCTC11435 (NZ_UHIG01000003.1) was included for perspective.

A Core-genome Multi Locus Sequence Typing (cgMLST) analysis of the 43 whole-genome sequenced *Vibrio cholerae* isolates was performed on PuBMLST with ReporTree (Mixão et al., 2023) using GrapeTree (Zhou et al., 2018) for visualization. The Minimum Spanning Tree (MST) was constructed based on the cgMLST 2443-loci PuBMLST schema (Liang et al., 2020). A threshold of seven allelic distances (ADs) was used, which is capable of identifying strains from the same outbreak and with potential epidemiological concordance (Liang et al., 2020). This same approach was used to check for the relatedness of the 43 *V. cholerae* isolates with other *V. cholerae* strains isolated in the world. However, in this case, a threshold of 20 ADs was used.

3. Results and discussion

The only pathogenic *V. cholerae* serogroups currently recognized as causing significant cholera outbreaks are O1 (primarily serotypes Inaba and Ogawa) and O139 (CODEX Alimentarius, 2010). The main virulence determinants of cholera, the co-regulated cholera toxin and pili, are part of horizontally transferred mobile genetic elements (Kumar et al., 2020).

While non-O1 and non-O139 *V. cholerae* (NOVC) strains are not typically associated with large-scale cholera pandemics, they can still cause disease. These strains are genetically diverse and can lead to various illnesses, including gastroenteritis, wound infections, ear infections and even bacteraemia, particularly in individuals with compromised immune systems or underlying health conditions (Trubiano et al., 2014; Chatterjee et al., 2009; Deshayes et al., 2015; Albuquerque et al., 2013). These facts lead these non O1/O139 *V. cholerae* strains to be considered of high relevance for public health in the European Union (EFSA Panel on Biological Hazards (BIOHAZ), 2024).

In this preliminary work, *V. cholerae* strains were detected and isolated from 43 of the 129 analyzed seafood raw samples (33.3 %) and were detected in 39.0 % of the shrimp samples (41/105) and 8.3 % (2/24) of the oysters' samples (isolates 36 and 37). It must be acknowledged that all shrimp samples, analyzed in this study, were purchased frozen. Freezing can reduce the viability of *Vibrio cholerae*, potentially leading to lower detection rates (Reily and Hackney, 1985). Therefore,

Table 2
Accession numbers and genome assemblies' quality information of the genomic sequences of the 43 whole-genome sequenced *V. cholerae* strains.

ID	Sample origin	ENA run accession ID	Assembly length (bp)	# contigs	N50	GC%
1	Unknown	ERR14205254	4012725	56	180780	47.39
2	Unknown	ERR14205226	4090500	131	63229	47.65
3	Unknown	ERR14205246	4181830	67	221670	47.56
4	Unknown	ERR14205235	3885070	51	224140	47.64
5	Unknown	ERR14205259	3903516	70	180799	47.63
6	Unknown	ERR14205256	3951631	79	148745	47.53
7	Unknown	ERR14205230	4003768	68	156398	47.41
8	Unknown	ERR14205257	3979385	86	172893	47.57
9	Ecuador	ERR14205243	4070434	73	155356	47.47
10	Ecuador	ERR14205265	3952097	87	136534	47.65
11	Ecuador	ERR14205247	4089130	76	164857	47.54
12	Ecuador	ERR14205248	4263156	87	224561	47.59
13	Ecuador	ERR14205240	4047050	88	170344	47.60
14	Ecuador	ERR14205253	4012561	71	178894	47.62
15	Ecuador	ERR14205228	4056589	83	116090	47.48
16	Ecuador	ERR14205264	4262448	87	173027	47.53
17	Ecuador	ERR14205260	4138254	117	72046	47.47
18	Ecuador	ERR14205231	4001877	68	119277	47.72
19	Ecuador	ERR14205225	4093697	51	176363	47.56
20	Ecuador	ERR14205242	4030540	61	157666	47.36
21	Ecuador	ERR14205244	4176535	108	107875	47.52
22	Ecuador	ERR14205251	4229951	71	164528	47.51
23	Ecuador	ERR14205262	4175385	66	143190	47.23
24	Ecuador	ERR14205232	4030894	57	165947	47.37
25	Ecuador	ERR14205267	4448854	76	132718	47.50
26	Unknown	ERR14205249	4211634	102	82782	47.40
27	Unknown	ERR14205255	3971439	82	122926	47.60
28	Unknown	ERR14205237	3914596	77	259476	47.64
29	Unknown	ERR14205261	4051949	69	180925	47.53
30	Unknown	ERR14205227	3988135	90	113936	47.51
31	Ecuador	ERR14205258	3979277	77	133615	47.53
32	India	ERR14205241	3979540	62	202270	47.62
33	India	ERR14205238	4193434	76	109282	47.54
34	India	ERR14205245	3833519	57	171337	47.78
35	India	ERR14205234	3895334	78	130760	47.65
36	Portugal	ERR14205236	4200529	81	120920	47.49
37	Portugal	ERR14205263	4115119	88	91147	47.47
38	Ecuador	ERR14205250	4015416	71	193441	47.51
39	India	ERR14205239	4049649	160	70764	47.48
40	Ecuador	ERR14205233	4019439	57	147821	47.39
41	Ecuador	ERR14205266	4029724	61	148295	47.38
42	Venezuela	ERR14205229	4077819	85	126738	47.55
43	Ecuador	ERR14205252	4007365	89	137855	47.69

the occurrence reported here might underestimate the true occurrence in fresh shrimp. It is also important to emphasize that oysters are typically consumed raw, which, if contaminated, increases the risk of occurrence of a potential outbreak.

All the isolates were determined to be NOVC strains, based on whole-genome sequencing (WGS), lacking the gene clusters encoding the somatic O1 and O139 antigens. It is worth mentioning that relying solely on WGS for *Vibrio cholerae* serotyping may present limitations as this approach predicts genetic potential but does not confirm phenotypic expression. The NOVC occurrence found in this study was higher than the ones reported in other studies where seafood samples were collected in Italy - 5.6 % (Ottaviani et al., 2009), in Dakar, Senegal - 1.6 % (Coly et al., 2013) and in Berlin - 6.3 % (Vu et al., 2018) but lower than one reported in Thailand- 44 % (Preeprem et al., 2014).

3.1. Virulence genes

Fig. 1 summarizes the results regarding the presence/absence of the tested *V. cholerae* main virulence genes on the 43 isolates.

It is important to note that, as expected, in this study, all sequenced *V. cholerae* strains seem to lack *ctxA*, *ctxB* (encoding cholera toxin- CT), *zot* (encoding zonula occludens toxin) and *ace* (encoding accessory cholera enterotoxin) genes, all genes part of the CTX genetic element (Faruque et al., 1998) of the filamentous bacteriophage (CTXϕ). The genetic exchange in the environment involving this bacteriophage allows the potential emergence of new *V. cholerae* toxigenic clones. These genetic exchanges are often influenced by environmental factors such as temperature, sunlight and osmotic conditions (Chowdhury et al., 2017). Besides the CTX genetic element, that carries cholera toxin genes, four pathogenicity islands have been detected in serogroups O1 and O139 of *V. cholerae*, associated with epidemic and pandemic cholera, *Vibrio* pathogenicity island-1 and 2 (VPI-1, VPI-2) and *Vibrio* seventh pandemic island-I and II (VSP-I, VSP-II) (Faruque and Mekalanos, 2003). In this study, none of the sequenced NOVC strains likely contain *acf* (accessory colonisation factor) and type IVb toxin co-regulated pilus (TCP) genes from the VPI-1. Moreover, the five VSP-I tested genes appear to be absent in all sequenced strains (Fig. 1). However, *nanH*, *nanK* and *nanA* genes, were detected in 41.9 % (18/43) of the *V. cholerae* strains sequenced in this study (Fig. 1). These genes are part of the VPI-2 and codify enzymes that allow pathogenic bacteria to utilize sialic acids as nutrient source, which can be crucial for their survival and virulence in host environments (Almagro-Moreno and Boyd, 2009a, 2009b). Moreover, in one of these strains (strain number 31) it was possible to detect a complete VPI-2.

It is also worth mentioning the presence of a partial VSP-II region in strain number 5 (Figure1). Fragments of the VSP-II have been identified in some clinical, seafood and environmental NOVC strains, characterized in other studies (Schwartz et al., 2019; Bhandari et al., 2023; Zhang et al., 2023). These fragments often include genes associated with virulence and survival in aquatic environments.

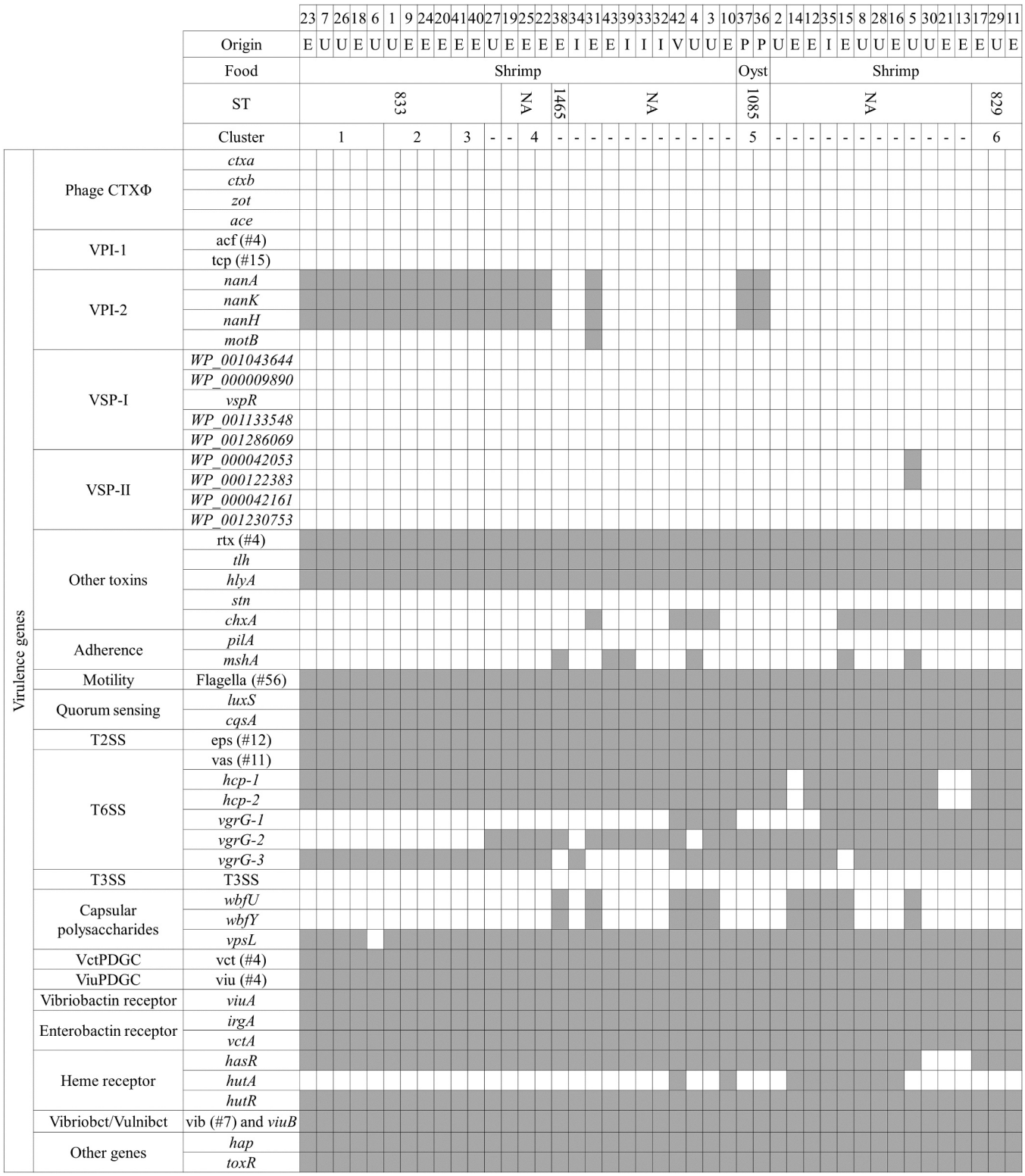


Fig. 1. Main virulence genes detected in the 43 *V. cholerae* sequenced isolates. Grey shadow means the gene is present (BLASTn; presence of the gene was considered when coverage and gene identity were higher than 70 % and 75 %, respectively). ST- Sequence type; NA- Not attributed; VPI- *Vibrio* pathogenicity island; VSP- *Vibrio* seventh pandemic island; T2SS-Type II secretion system; T3SS- Type III secretion systems; T6SS- Type VI secretion system; Vibriobct- Vibriobactin; Vulnibct- Vulnibactin; Oyst- Oysters; Origin: E- Ecuador; I- India; P-Portugal; V- Venezuela; U- Unknown. #- number of different genes which presence was evaluated. Complete information on Supplementary Table 1.

Concerning the presence of other toxins besides the ones that are codified by the genes of the CTX genetic element, it is worth of noting that it was not possible to detect the presence of the *stn* gene, encoding the heat-stable enterotoxin, in any of the tested strains. However, *rtxa, rtxb*, *rtxc*, *rtxd*, encoding Multifunctional autoprocessing RTX toxin (MARTX), *tlh,* encoding thermolabile hemolysin (TLH), and *hlyA*, encoding *V. cholerae* cytolysin (VCC) were detected in all the tested strains (Fig. 1). MARTX, TLH and VCC are *V. cholerae* exotoxins that play significant roles in its pathogenicity (Kim, 2020). Furthermore, the cholix toxin gene, *chxA* was detected in 34.9 % (15/43) of the sequenced NOVC strains. This toxin is an ADP-ribosylating enzyme that targets Eucariotic elongation factor 2 (eEF2), leading to inhibition of protein synthesis and cell death and has been detected in NOVC from seafood and clinical strains by other authors (Awasthi et al., 2013; Tangestani et al., 2020; Zhang et al., 2023).

Regarding the presence/absence of the type IVa Chitin-regulated pilus (ChiRP), we observed that all the tested strains contain *pilB*, *pilC*, *pilD/vcpD* genes but none of them, apparently, contains the *pilA* gene, encoding fimbrial protein PilA (BLASTn results with high identities (>85 %), but low query coverages (<40 %) (Fig. 1, Supplementary Table 1). ChiRP, or DNA-uptake pilus, seems to promote inter-bacterial interactions during chitin surface colonisation allowing bacteria to take up DNA from their environment (Ellison et al., 2018). Contrarily to disease-causing pandemic *V. cholerae* strains, which typically encode the same major pilin subunit, PilA, the environmental isolates have extensive strain-to-strain variability in PilA, enabling cells producing pili composed of different PilA subunits, assisting bacterial kin recognition (Adams et al., 2019). Considering that, in this study, all sequenced strains were classified through WGS as non-O1 and non-O139, generally considered environmental strains, it can justify the results obtained for *pilA* gene.

V. cholerae, uses their flagella and mannose-sensitive hemagglutinin (MSHA) type IVa pili synergistically to switch between two motility states that facilitate surface selection and attachment (Utada et al., 2014; Zhang et al., 2021). In this study, all isolates contain the 56 tested flagella-related genes. Moreover 93 % (13/14) of the tested MSHA pili-related genes were detected in all isolates, and the *mshA* gene, encoding the structural subunit of mannose sensitive hemagglutinin pili was also detected in 6 of the isolates (isolates 4, 5, 15, 38, 39 and 43) (Fig. 1; Supplementary Table 1).

V. cholerae uses cell-to-cell communication to control pathogenicity and biofilm formation. This process, known as quorum sensing, relies on the secretion and detection of two major signalling molecules called autoinducers, CAI-1 and AI-2, that function synergistically to control gene regulation (Higgins et al., 2007). The genes *cqsA* and *luxS* encoding synthases of the two autoinducers were found in 100 % of the sequenced genomes.

The type II Secretion System (T2SS) in *V. cholerae* is a multi-protein complex that transports fully folded proteins from the periplasmatic space across the bacterial outer membrane into the extracellular environment. This system is crucial for the secretion of cholera toxin, a major virulence factor of *V. cholerae* (Sandkvist et al., 1997; Reichow et al., 2010; Johnson et al., 2006). The VAS T6SS (Vibrio Antagonistic System Type VI Secretion System) in *V. cholerae* is a complex mechanism used by bacterium to inject toxic effector proteins into neighbouring cells. This mechanism is used both in competition and virulence (Lien and Lai, 2017). The analysis revealed that all the sequenced strains are probably equipped with a functional type II Secretion System (T2SS), since all the 12 tested genes of this system were found in all the strains. Moreover, the 11 tested genes of the core region of *Vibrio* Antagonistic System Type VI Secretion System, were present in all isolates and at least two of the five genes encoding VAS T6SS secreted effectors were present in all the tested strains (Fig. 1, Supplementary Table 1). In opposition, Type III secretion system (T3SS) was not detected in any of the NOVC strains sequenced in this study (Fig. 1). The T3SS translocates a number of effectors to the host cell which interfere with host cell signalling pathways (Alam et al., 2011). A functional T3SS has been shown to be crucial for the pathogenicity of the NOVC strain AM-19226 (Shin et al., 2011).

Polysaccharide capsules are critical elements that protect bacteria against environmental and host factors, including the host immune system (Hsieh and Allen, 2020). In this study, it was possible to detect 6 of the 9 tested capsular polysaccharide genes in all the sequenced strains (Supplementary Table 1). Moreover, only one strain (strain 6) appears not to possess the *vpsL* gene, which encodes the capsular polysaccharide biosynthesis glycosyltransferase. Regarding *wbfU* and *wbfY* genes, encoding galactosyl-transferase and mannosyl-transferase, respectively, it seems that the presence of one is associated with the presence of the other. These genes were detected in 10/43 (23.3 %) of the sequenced strains (Fig. 1, Supplementary Table 1).

The genes coding for VctPDGC and ViuPDGC systems, both important iron transport mechanisms of *V. cholerae* (Wyckoff and Payne, 2011), as well the ones related with the synthesis and transport of the two used siderophores, vibriobactin and enterobactin (Wyckoff and Payne, 2011), were detected in all the strains. Moreover, from the 3 tested genes related with TonB-dependent transport of heme, other system for iron acquisition (Mey and Payne, 2001), *hutR* was detected in all *V. cholerae* isolates, *hasR* in 40/43 (93.0 %) and all the three genes (*hutR*, *hasR* and *hutA*) in 9/43 of the isolates (20.9 %).

The *hap* gene in *V. cholerae* encodes the hemagglutinin protease (HAP), which is crucial for the bacterium's pathogenicity, helping in degrading host proteins, facilitating tissue invasion and colonization. It also plays a role in disrupting the intestinal barrier, contributing to the severe diarrhoea seen in cholera (Syngkon et al., 2010). In this study, this gene was detected in all the sequenced strains. Also, *toxR gene*, coding a transcription factor that controls the expression of several virulence factors (Morgan et al., 2019), was detected in all the isolates.

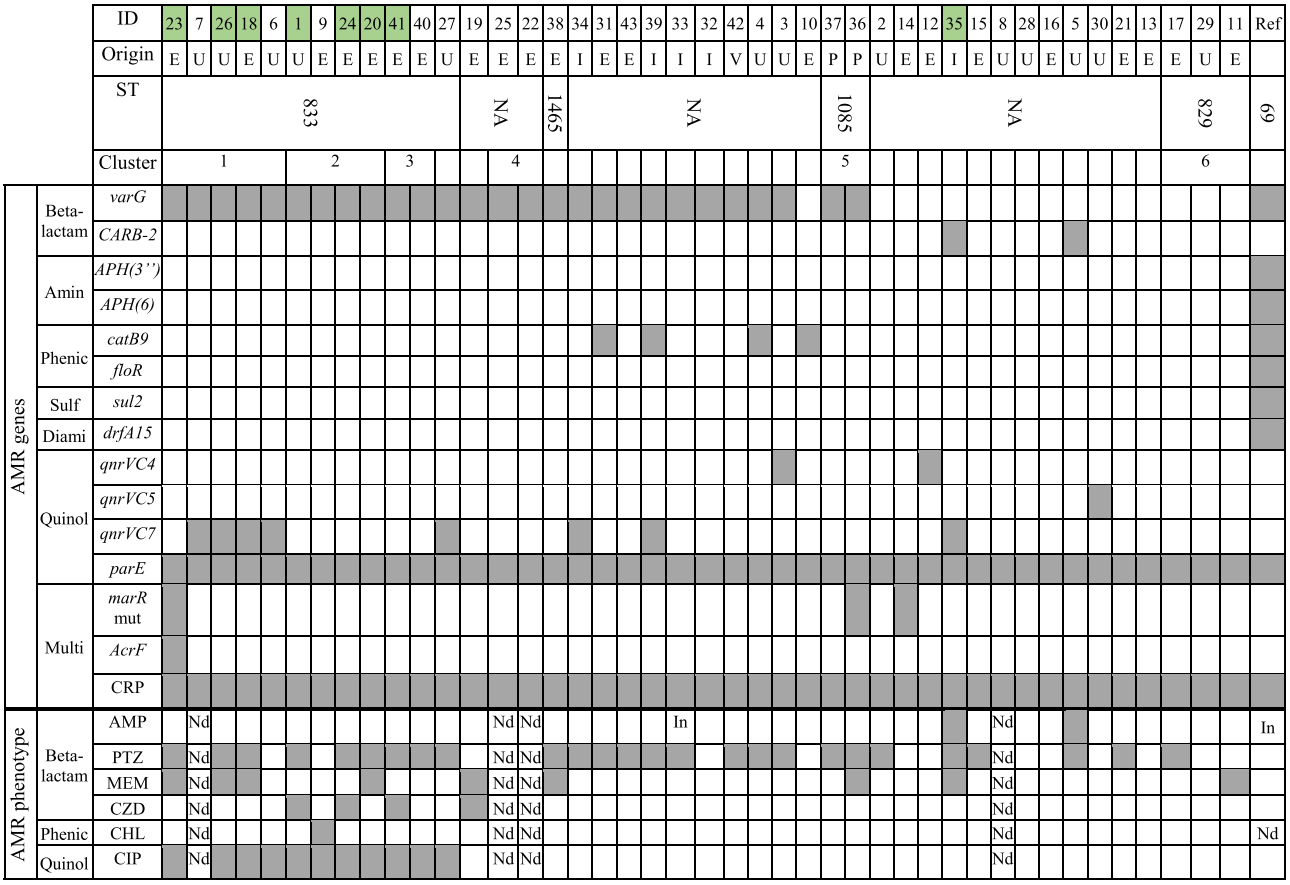
In conclusion, the analyses revealed that the main cholera virulence factors and accessory virulence factors of the toxigenic O1/O139 *V. cholerae* strains seem to be absent in all the studied strains. This study is descriptive in nature, and the absence of detection of major cholera virulence determinants suggests that these strains possess genetic characteristics typical of environmental, non-epidemic populations rather than clinical, disease-causing strains. However, the studied strains appear as a potential significant reservoir of virulence and fitness genes, many of them already found in NOVC clinical strains. This fact raises the possibility of the emergence of environmental bacteria with new virulence traits that might constitute a risk for human health. In fact, in recent years, the number of foodborne infections with non-O1 and non-O139 *Vibrio cholerae* (NOVC) has increased worldwide. These have ranged from sporadic infection cases to localized outbreaks. The majority of case reports describe self-limiting gastroenteritis. However, severe gastroenteritis and even cholera-like symptoms have also been described (Zhang et al., 2024).

3.2. Antibiotic resistance (AMR) genes

Antibiotics are powerful, lifesaving drugs used to fight infections caused by bacteria or fungi. However, antimicrobial resistant bacteria pose one of the most significant healthcare challenges, with an estimation of being directly responsible for 1.27 million global deaths in 2019 and contributed to 4.95 million deaths (Antimicrobial Resistance Collaborators, 2022).

In this study, several known antimicrobial resistance (AMR) genes were detected in the sequenced *V. cholerae* strains. The most abundant AMR genes were CRP and *parE*, detected in 100 % of the isolates (Table 3). CRP is a global regulator of multidrug resistance, mainly described in *E. coli* (Nishino et al., 2008), presenting sequence variants in the resistomes of several gram-negative bacterial species (https://card.mcmaster.ca/ontology/36657). Gene *parE* encodes a subunit of DNA topoisomerase IV; mutations in this gene can alter the target site of fluoroquinolone antibiotics, reducing their effectiveness (Nawaz et al., 2015; The Comprehensive Antibiotic Resistance Database (https://card.

Table 3
Antibiotic resistance genes and antibiotic resistance phenotypes detected in 43 and 39 *V. cholerae* isolates, respectively.



Grey shadow means positive (presence of the gene/ phenotypic resistance) and green shadow on ID number means the strain was classified as Multidrug resistant (MDR). **NA**- Not attributed; **Nd**- Not determined; **In**- Intermediate resistance; **Ref**: *Vibrio cholerae* O1 str 2010EL-1786 (ATCC BAA-2163); **ST**- Sequence Type; **Multi**- Multiple; **AMR-** Antimicrobial resistance; **Amin**- Aminoglycoside; **Phenic**- Phenicol; **Sulf**- Sulfonamide; **Diami**- Diaminopyrimidine; **Quinol**- Quinolone; **AMP-** Ampicillin**; CHL-** Chloramphenicol**; CIP-** Ciprofloxacin; **CZD**- Ceftazidime **MEM-** Meropenem; **PTZ**- Piperacillin-Tazobactam; Origin: **E**- Ecuador; **I**- India; **P**-Portugal; **V**- Venezuela; **U**- Unknown. For detailed information about disk diffusion assay results, please see Supplementary Table 2)

mcmaster.ca/)). The v*arG* gene, which is known to have β-lactamase activity against penicillins, cephalosporins and carbapenems (Lin et al., 2017) was detected in 27/43 (62.8 %) of the isolates (Table 3). A high prevalence of the *v*arG gene was also reported in a study conducted on various *Vibrio* species isolated from human, food, animal and environmental sources in Latin America (Vilela FP and Falcão, 2021). Additionally, two isolates (5 and 35) carried *CARB-2* gene, a determinant of beta-lactam resistance (Petroni et al., 2004), four isolates carried *catB9*, a determinant of phenicol resistance (Heidelberg et al., 2000; The Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/)) and eleven isolates carried variants of *qnrVC4, qnrVC5* and *qnrVC7*, determinants of quinolone resistance (Xu et al., 2023) (Table 3).

It is also important to highlight the presence of a mutation in *marR* gene, which is associated with antibiotic resistance, in three strains, and the presence of *AcrF* gene in one. The *marR* gene in *Vibrio cholera* encodes a regulatory protein that plays a crucial role in antibiotic resistance and stress response (Beggs et al., 2020). Mutations in the *marR* gene can lead to changes in the expression of multiple genes, including those involved in efflux pumps and other resistance mechanisms. The *AcrF gene* in *Vibrio cholerae* is associated with antibiotic resistance. This gene is part of the AcrAB-TolC efflux pump system, which helps bacteria expel toxic substances, including antibiotics, from their cells (Weston et al., 2018; Andersen et al., 2015).

In this work there seems to be some agreement between the presence of the *v*arG and CARB-2 genes and the beta-lactam resistance phenotype. In fact, only three of the 26 phenotypically tested strains (11.5 %) containing at least one of these genes did not present a resistance phenotype to any of the beta-lactams antimicrobials tested (strains 6, 9 and 32). In contrast, eight out of the 13 (61.5 %) phenotypically tested strains that did not contain v*arG* or CARB-2 genes did not present a resistance phenotype to any of the beta-lactams antimicrobials tested (strains 10, 12, 13, 14, 16, 28, 29, 30). Moreover, it seems that the presence of CARB-2 gene is related with ampicillin resistance, since only the two strains containing this gene (strains 5 and 35) presented phenotypic resistance to ampicillin (Table 3). However, *catB9* presence does not appear to be associated with chloramphenicol resistance as the only strain phenotypically resistant to this antimicrobial (strain 9) seems not to contain this gene and the four strains where this gene was detected (strains 4, 10, 31 and 39) did not show resistance (Table 3). Furthermore, there also appears to be no association between the presences of the *qnrVC4*, *qnrVC5*, *qnrVC7* and *parE* genes and the resistance to quinolones, as there are several strains containing these genes without showing resistance.

The divergences between the phenotypic and genome-based antimicrobial resistance assessment, found in this study, were in accordance with the ones found by others (Lepuschitz et al., 2019; Schmidt et al., 2023), showing that, for NOVC strains, resistance databases are currently inappropriate for an assessment of antimicrobial resistance.

In this work, it is important to emphasize the high proportion of *Vibrio cholerae* isolates resistant to at least one of the tested

antimicrobials (30/39, 76.9 %), with eight of the tested strains being characterized as multidrug-resistant (8/39, 20.5 %). These results support the ones obtained by others (Lepuschitz et al., 2019; Schmidt et al., 2023; Vilela and Falcão, 2021) confirming that NOVC environmental strains can carry important AMR markers that may difficult treatment options for infections caused by these bacteria, and, eventually, transfer resistance to other pathogenic strains, making it crucial to monitor and understand their resistance patterns.

3.3. Phylogenetic and clustering analysis

In order to assess the genetic relatedness among the 43 *V. cholerae* isolates, a phylogenetic tree and a core-genome clustering analysis were performed (Figs. 2 and 3, respectively). The phylogenetic tree and the cgMLST analysis of the 43 *Vibrio cholerae* strains were concordant, both revealing distinct clustering patterns that reflect genetic diversity among the studied isolates.

The cgMLST analysis revealed six genetic clusters of high closely related isolates (≤ 7 Allelic Differences). Clusters 1, 2 and 3, comprise five, four and two isolates, respectively, all with ST833. Cluster 4 includes two strains with an unknown ST; Cluster 5 comprises two isolates with ST1085 and cluster 6 includes three isolates with ST829 (Fig. 3). As expected, with the exception of isolate 6 which is the only one from cluster 1 that likely do not contain the vpsL gene, isolates that belong to the same cluster possess similar virulence profiles (Fig. 1). Furthermore, isolates from the same cluster are from samples with similar origins, five of the six clusters comprise samples that had origin in Ecuador or that have unknown origin, and one (cluster 5) includes samples produced in Portugal (oysters) (Figs. 1 and 3).

In order to access the genetic similarity among the 43 NOVC isolates and other *V. cholerae* strains isolated globally, we have decided to perform a core-genome clustering analysis comprising the 43 NOVC isolates and the ones available in PuBMLST that were deposited by other European countries as well as the ones submitted by India, Ecuador and Venezuela (countries of origin of the samples from where the 43 NOVC were isolated) (Fig. 4).

This analysis included 207 isolates submitted to PuBMLST, by ten countries (India-113; Portugal-43; Switzerland- 23; Ukraine- 17; Sweden −3; United Kingdom- 3; Spain- 2; Germany- 1; Hungary- 1 and Romania-1) and isolated from 3 different sources (151 human isolates; 10 environmental, the 43 studied within the scope of this work, that were isolated from seafood, and 3 had a missing source). Despite the evident genetic variability among the strains included in this cgMLST analysis, it was possible to observe a high genetic proximity among some strains

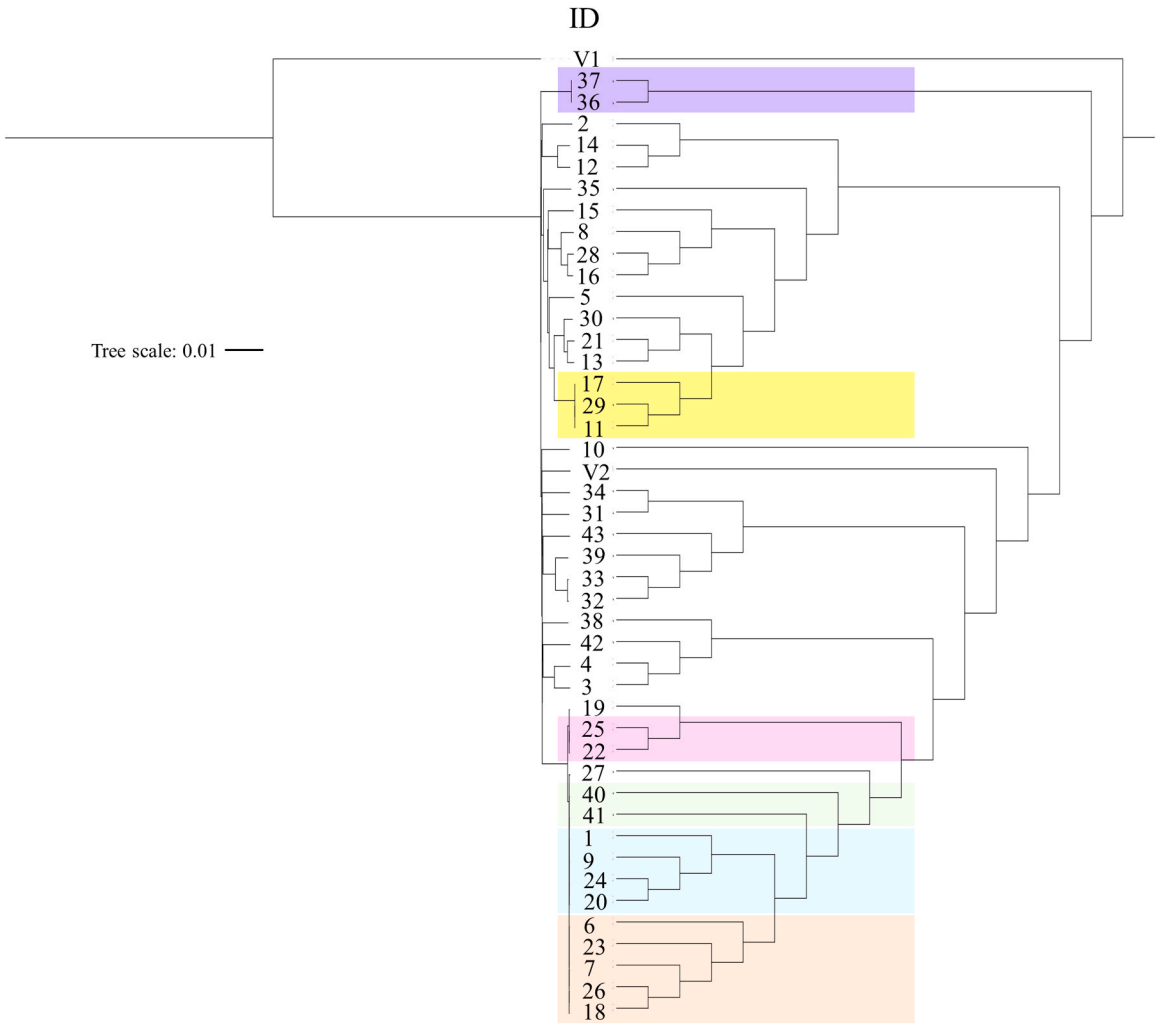


Fig. 2. Phylogenetic tree of the *Vibrio cholerae* isolates (n = 43).
The Phylogenetic tree was constructed based on a 2457 loci PuBMLST schema (all loci). The tree was rooted on *V. cholerae* O1 biovar El Tor str. N16961 (V2) and a sequence of *Vibrio mimicus* strain NCTC11435 was included for perspective (V1). On the left side it is presented a phylogenetic tree where branch lengths were used and on the right side it is presented a phylogenetic tree where branch lengths were ignored. Isolates belonging to the same cluster (cgMLST allelic distances ≤7) are highlighted with the same colour.

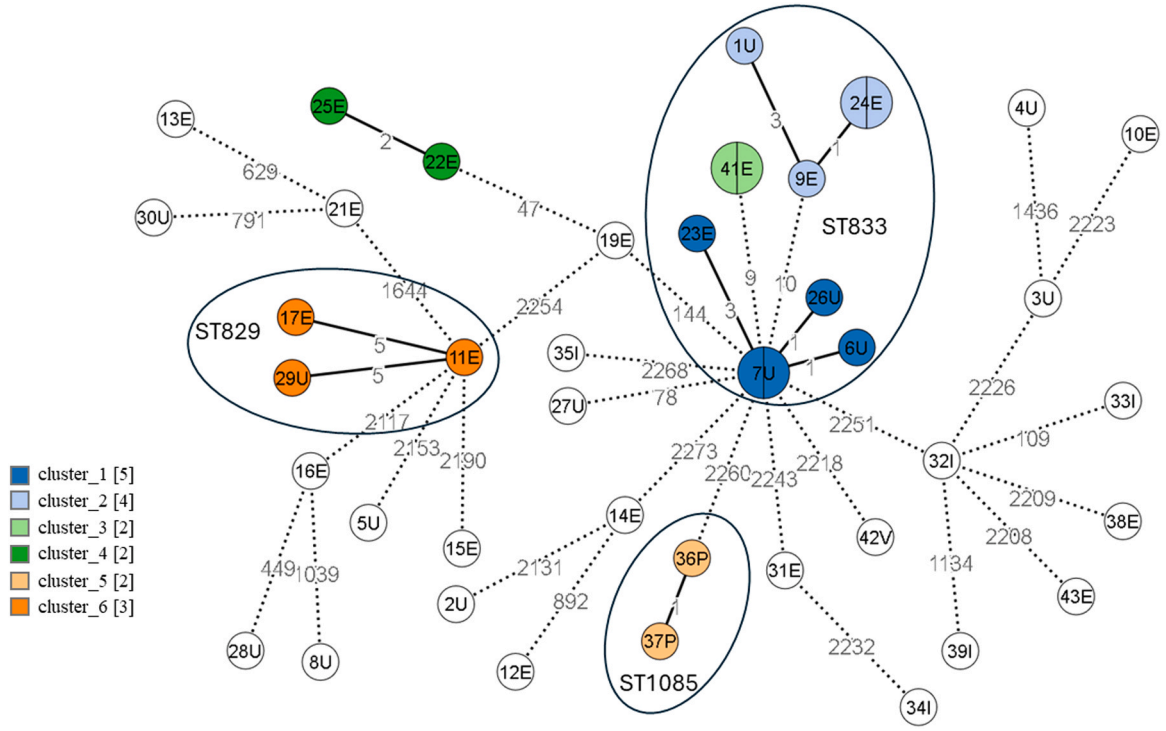


Fig. 3. Minimum Spanning Tree (MST) of *Vibrio cholerae* NOVC isolates (n = 43).
The Minimum Spanning Tree (MST) was constructed based on the cgMLST 2443-loci PuBMLST schema. Each circle represents one isolate and numbers between nodes indicate the allelic distances (ADs) between the isolates. Straight and dotted lines reflect nodes linked with ADs below and above a threshold of seven, respectively. The information in each node corresponds to the number of the isolate and its origin (E- Ecuador; I- India; P- Portugal; V- Venezuela; U- Unknown). Data visualization was performed by using GrapeTree dashboard (Zhou et al., 2018) with the node colours reflecting the clusters (allelic distances ≤7) (Liang et al., 2020).

isolated in different countries (India and Ukraine- cluster 11) (Fig. 4), and isolated from different sources (among Ukraine isolates, from cluster 11, there are strains isolated from environmental and human sources). These observations reinforce the importance of a One Health approach, showing that a better understanding of *V. cholerae* isolates from different countries and sources, particularly from seafood and aquatic environments, is essential for a better understanding of the epidemiological links between all the involved sources of infection.

4. Conclusion

In this study we found a high prevalence (33.3 %) of *V. cholerae* strains, classified through WGS as non-O1/non-O139, in seafood samples available in the Portuguese market. Although most of the analyzed positive samples were produced in non-European countries, it was possible to isolate *V. cholerae* from two samples of oysters produced in portuguese coastal areas. While the studied strains were all classified as non-O1/non-O139, and seem to lack the main cholera virulence factors of the CTX phage element, they are highly diverse genetically andcarry diverse virulence factors, similar to those found in the *V. cholerae* serogroups O1 and O139 strains and/or in NOVC clinical strains. Furthermore, they showed resistance to several antimicrobials, with eight of them being classified as multidrug-resistant. The performed core genome analysis reinforces the idea that the One Health approach is crucial for detecting and managing foodborne outbreaks. Being aware that a proportion of the seafood commercialized in Europe come from third countries, some of them where cholera may be endemic, and that it is expected a rise of *Vibrio* spp. abundance in European coastal waters, due to climate change, an increase in infections caused by these bacteria is predictable. It is thus crucial to develop effective monitoring systems to prevent *Vibrio* spp. infections in Europe, thereby promoting food safety and protecting public health.

Authors' contribution

The corresponding author ensure that no inappropriate co-authors are included on the paper, and that all co-authors have seen and approved the final version of the paper and have agreed to its submission for publication.

Institutional review board statement

Ethical review and food samples available in the Portuguese market.

CRediT authorship contribution statement

Carla Maia: Writing – review & editing, Methodology. **Maria João Barreira:** Writing – review & editing, Methodology. **Cristina Pintado:** Writing – review & editing, Supervision, Conceptualization. **Cristina Belo Correia:** Writing – review & editing, Validation. **Rita Batista:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis. **Teresa Lopes:** Writing – review & editing, Methodology. **Margarida Saraiva:** Writing – review & editing, Validation, Supervision, Conceptualization. **Isabel Bastos Moura:** Writing – review & editing, Methodology. **David Lopes:** Writing – review & editing, Methodology. **João Rodrigues:** Writing – review & editing, Methodology. **Isabel Sousa:** Writing – review & editing, Methodology. **Camila Fernandes:** Writing – review & editing, Methodology.

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Declaration of Competing Interest

The authors declare that they have no known competing financial

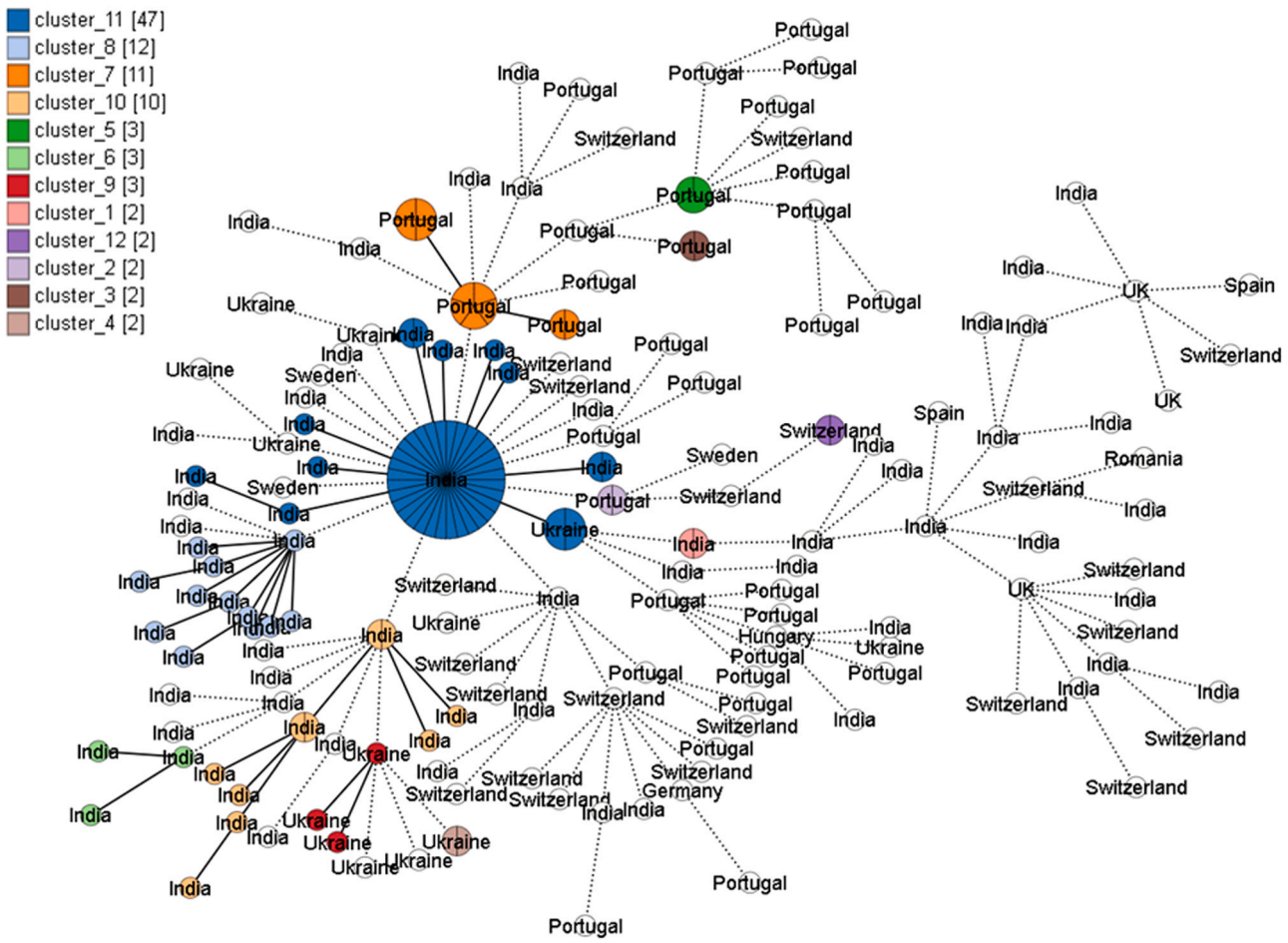

Fig. 4. Minimum Spanning Tree (MST) of *Vibrio cholerae* isolates (n = 207).
The Minimum Spanning Tree (MST) was constructed based on the cgMLST 2443-loci PuBMLST schema. Each circle (node) represents one isolate. Each division in a node corresponds to a single isolate. Branches with ≤ 7 allelic distances (ADs) were collapsed. Straight and dotted lines reflect nodes linked with ADs below and above a threshold of 20 ADs, respectively. The information in each node corresponds to the country where the strain was isolated (the 43 NOVC isolates sequenced within the scope of this work are the ones from Portugal). Data visualization was performed by using GrapeTree dashboard (Zhou et al., 2018) with the node colours reflecting the clusters (allelic distances ≤20).

interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2025.100636.

Data availability

I have shared the link to my data

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