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# Genotypic assessment of a dichotomous key to identify *Vibrio coralliilyticus*, a coral pathogen

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ABSTRACT: *Vibrio coralliilyticus* is a known pathogen to corals and larvae of bivalves. Its identification is made based on phenotypic and genotypic characters of isolated strains. To evaluate the efficiency of the phenotypic identification, 21 strains identified as *V. coralliilyticus* using a widely used dichotomous key were analyzed by qualitative PCR and sequencing of the 16S rDNA region. The results obtained by the behavioral test, amino acids usage, allow us to distinguish 3 A/L/O profiles: (1) A+/L–/O+; (2) A+/L+/O+; and (3) A–/L+/O+. In the genotypic tests, all strains tested positive with primers specific for the *Vibrio* genus. However, when primers were used for species identification, the results did not match those obtained with the dichotomous key chosen. The phenotypic characteristics taken into account to set apart *V. coralliilyticus* and other species were not proven to be efficient. More information about the morphological diversity of colonies and enzymatic activities should be considered in the formulation of phenotypic keys for *V. coralliilyticus* and related species.

KEY WORDS: Vibriosis · Coral disease · Phenotype · Genotype



## INTRODUCTION

Coral reefs are one of the most diverse ecosystems and present the highest primary production rates in marine environment. However, natural and anthropic activities have been compromising the health of those ecosystems in the last years, especially with the impact of diseases related to tissue loss, collectively called white syndrome (WS) (Wilson et al. 2013, Séré et al. 2013). Although the role of bacteria in the health of these organisms is yet to be fully understood, vibrios are known as an important part of the coral reef microbiota. Some species work in mutualistic relationships with corals, producing nutrients and secondary metabolites (Munn 2015), whereas other species have great influence in unbalancing the health of those organisms (Ushijima et al. 2013, 2014, Pollock et al. 2015).

Among the species from the *Vibrio* genus, *Vibrio coralliilyticus* is a prominent pathogen, causing cellular lysis and bleaching the corals. It has been detected in large amounts in bleached *Pocillopora damicornis* corals collected in the Red Sea and Indian Ocean (Ben-Haim et al. 2003). This bacterium has also been identified as the etiological agent responsible for vibriosis in bivalve larvae of various species worldwide (Richards et al. 2015).

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Classical microbiological methods as well as molecular biological DNA-based diagnostic ones are used to identify *Vibrio* species. The traditional methods of bacterial identification are based on morphological and physiological characteristics such as macroscopic and microscopic morphology, spore formation, enzyme production and the fermentation of different carbohydrates, production of gas, and sensitivity to antibiotics. Among the classical methods, the dichotomous key based on biochemical tests, proposed by Noguerola & Blanch (2008), is widely used in laboratories (Laganà et al. 2011, Albuquerque et al. 2013, Silvester et al. 2017). Other methods are also used: the API system (BioMerieux) and Biolog are both based on the fermentation patterns presented by the microorganisms (Ben-Haim et al. 2003, Moraes et al. 2013). As for molecular techniques, polymerase chain reaction (PCR) is used in the identification and confirmation of *Vibrio* species, serogroups, biotypes and virulence factors (Schirmeister et al. 2014, Kim et al. 2015) and DNA sequencing (Letchumanan et al. 2014). Today, there are endless molecular tools used to accurately identify bacterial isolates. Now, the methods used are not limited by their level of performance but rather by their cost, suggesting a different tool might be adequate for different purposes.

The purpose of the present study was to analyze the efficiency of a dichotomous key based on phenotypic tests for the identification of *V. coralliilyticus* species from various sources. For the evaluation, molecular biology techniques, such as qualitative PCR and sequencing of the bacterial 16S RNA gene, were used.

## MATERIALS AND METHODS

### Culture sources

Isolates were taken from estuary waters and the tissue of *Litopenaeus vannamei* shrimp cultivated in the state of Ceara, Brazil. Twenty-one strains were analyzed and identified using the dichotomous key proposed by Noguerola & Blanch (2008), based on the behavior of the isolates in tests of arginine hydrolysis, lysine, and ornithine decarboxylase.

The strains belong to the collection of the Laboratory of Environmental and Fishery Microbiology (LAMAP), Sea Sciences Institute (LABOMAR), at Federal University of Ceara. Isolates were stored at −20°C in tryptone soy broth (TSB; Difco) with 20% of glycerol. Strains were reactivated in TSB with 1% NaCl and, after incubation at 35°C for 18 h, were evaluated for purity. At this stage, Gram staining and plating in thiosulphate citrate bile salt sucrose agar at 35°C were conducted.

Several (2–3) typical sucrose-positive colonies were selected and transferred to tryptone soya agar (Difco) tubes with 1% of NaCl, inclined and incubated for 18 to 24 h at 35°C.

### Vibrio DNA extraction and amplification

The total DNA of the cultures was extracted using the Wizard® Genomic DNA Purification Kit (Promega) kit. The product of the extraction was used as target DNA for primers specific to the genus *Vibrio* (VibF-727 and VibR-1423) (Sousa et al. 2006) and for the species *V. coralliilyticus* (76F and 1019R) (Poulson et al. 2008). Primers used are described in Table 1.

In all amplifications, 2 strains were used as controls: a reference strain, *Vibrio cholerae* non-O1 IOC 15177 (P), provided by the Owaldo Cruz Institute (FIOCRUZ-RJ), and a *Vibrio mimicus* strain, from the LAMAP bacterial collection.

Products of DNA extraction and PCR were subject to the gel electrophoresis method in 1% agarose gel with the addition of GelRed for the visualization of the amplified material in a transilluminator (Espectroline-UV) with ultraviolet light. Runs were made in an agarose gel slab 7 cm wide and 14 cm long, at 120 V and 500 mA for 60 min. The images obtained from the gel were digitalized in the Kodak EDAS290 photodocumentation system. A 1000 bp DNA ladder (Sigma) was used as a standard reference for amplicon size.

### Identification by 16S rDNA gene sequencing

Strains identified by the classical method and molecular biology were chosen for sequencing. The bacteria rRNA 16S gene was amplified using the oligonucleotide pair U968 (5′-AAC GCG AAG AAC CTT AC-3′) and L1401 (5′-CGG TGT GTA CAA GAC CC-3′), generating a fragment of 433 bp of the target gene (Nübel et al. 1996).

The 16S amplicons were sequenced using an automated capillary sequencer (ABI 3500) following the method described by Sanger et al. (1977). The obtained sequences were analyzed with the software MEGA v.6 (Tamura et al. 2013) with a later comparison to the sequences archived in GenBank using the

Table 1. Primer sequences used in the detection of isolates of *Vibrio* spp. and *V. coralliilyticus* (*Vc*). bp: base pair

| Target | Primer | Sequence (5′–3′) | Themocycling conditions | Amplicon size (bp) | Source |
|---|---|---|---|---|---|
| *Vibrio* spp. | VibF-727:<br>VibR-1423: | AGG CGG CCC CCT GGA CAG A<br>RCT TCT KKT GCA GCC CAC TCC CA | Initial denaturation:<br>94°C for 2 min<br>30 cycles:<br>94°C for 1 min<br>50°C for 1 min<br>72°C for 2 min<br>Final extension:<br>72° for 8 min | 696 | Sousa et al. (2006) |
| *Vc* | 76F:<br>1019R: | GTT RTC TGA ACC TTC GGG GAA CG<br>CTG TCT CCA GTC TCT TCT GAG G | Initial denaturation:<br>95°C for 5 min<br>30 cycles:<br>95°C for 30 s<br>67°C for 30 s<br>72°C for 1 min<br>Final extension:<br>72° for 10 min | 940 | Poulson et al. (2008) |

Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997).

## RESULTS AND DISCUSSION

*Vibrio coralliilyticus,* accepted as a species of the *Vibrio* genus since 2003, is recognized as a pathogenic agent to corals (Wilson et al. 2013). Environmental temperature regulates the expression of bacterial virulence factors of this *Vibrio* species, making its research and identification fundamental in tropical ecosystems (Kimes et al. 2012).

The dichotomous key proposed by Noguerola & Blanch (2008) is useful in the identification of most *Vibrio* species. The process is based on a matrix of phenotypic results, which, using fewer tests, favor a more discriminate choice of a profile compatible with the characteristics of vibrio isolates. The basic reference tests in the key are the decarboxylation of lysine (L) and ornithine (O) and arginine hydrolysis (A). Other researchers confirmed the reliability of this key for *V. cholerae* (Menezes et al. 2014) and *V. parahaemolyticus* (Croci et al. 2007), in comparison to molecular identification methods.

In the analysis of the identification scheme of the 21 isolates compatible with *V. coralliilyticus* identified by the Noguerola & Blanch (2008) key, the results obtained in the amino acid decarboxylation tests, yielded 3 different A/L/O profiles: (1) A+/L–/O+; (2) A+/L+/O+; and (3) A–/L+/O+ (Table 2). These results consequently implied different profiles that were oriented to specific keys within the identification scheme.

In one of these profiles (A+/L–/O+) in the Noguerola & Blanch Key, the only test that distinguishes between *V. mimicus* and *V. coralliilyticus* is the latter's capacity to grow in NaCl concentrations below 1%, pointing to a high phenotypic similarity between the species. Bacterial growth in the absence of NaCl can be influenced by the conditions of the inoculum, its origin and confirmation time that can to lead to false interpretations in the identification of isolates.

Hidalgo et al. (2008), in a study of the diversity of *Vibrio* spp. in clams, using the key proposed by Noguerola & Blanch (2008), have verified that the results in growth tests varied according to temperature and NaCl concentration, as in the Voges Proskauer tests. Undoubtedly, the phenotypic characterization in the key is not enough to identify strains to the species level. It is supposed that this difference stems from the widespread use of clinical strains instead of environmental strains as the standard in the classification of vibrios.

Table 2. Strain grouping according to the use profile of arginine, lysine and ornithine. A: Arginine hydrolysis; L: lysine decarboxylase; O: ornithine decarboxylase

| A/L/O profile | Strain code(s) |
|---|---|
| A+/L–/O+ | 42 |
| A+/L+/O+ | 7, 13, 15, 22, 26, 29, 31, 41, 47, 57, 62, 70, 71, 75, 77 |
| A–/L+/O+ | 32, 49, 63, 74, 76 |

The phenotypic identification of vibrios is problematic due to the high variability of biochemical characteristics and the narrow genomic similarities between many species. Other microbiologists have pointed out deficiencies in the taxonomic schemes based on phenotypic tests, since distinct species might have similar profiles (Amaral et al. 2014). In this respect, the species that present the highest phylogenetic similarity with *V. coralliilyticus* are *V. tubiashii* (97.2%), *V. nereis* (96.8%) and *V. shilonii* (96.6%), according to Ben-Haim et al. (2003). Amaral et al. (2014) propose that *V. coralliilyticus* and *V. tubiashii* might be considered sister species and add *V. brasiliensis*, as they present highly similar genomes (~70%) and almost indistinguishable phenotypes.

In genotypic tests, when primers specific for the *Vibrio* genus were used, all 21 strains were confirmed, demonstrating that the previous isolation and tests were efficient. However, when primers were used for species identification, the results did not match those based on the dichotomous key. This led us to suppose that the key was not efficient for the species due to the high false positive percentage.

Pollock et al. (2010) found a high level of genetic polymorphism between *V. coralliilyticus* strains displaying similar phenotypic characteristics, and emphasized the need for a multilocus approach to infer the phylogeny of *V. coralliilyticus.* Kim et al. (2017) reported the complete genome of *V. coralliilyticus* Strain 58, which was originally isolated from inactive Pacific oyster *Crassostrea gigas* larvae in Japan. The assembled genome consisted of 2 chromosomes and 1 plasmid. The high genetic diversity displayed and the lack of a consistent lineage suggest that the species does not possess a strong clonal structure. In opposition, Rodríguez-Camacho et al. (2014), while analyzing samples of *V. parahaemolyticus* and *V. harveyi* isolated from shrimps, acknowledge a 100% efficiency in the species confirmation by genotyping.

Phenotypic classification of specific microorganisms should be complemented by genotypic analysis in order to differentiate the species completely (Amaral et al. 2014). Biochemical tests before molecular ones act as a filter in the identification of strains pertaining to the *Vibrio* genus (Gomez et al. 2013).

According to Poulson et al. (2008), the primer pair used to test strains deemed to be *V. coralliilyticus* is highly specific, especially in environmental samples, as the primers suffer only slight degeneracy, building confidence in the identification of this coral reef pathogen.

Molecular biology techniques are being used more often. They improve bacterial identification and classification, particularly when genotypic methods are aimed at DNA and RNA, notably including PCR, amplification of specific genetic segments and identification of DNA polymorphism (Donelli et al. 2013).

After sequencing, 17 strains were confirmed as belonging to the *Vibrio* genus: *V. alginolyticus* (13), followed by *V. cholerae* (2), *V. parahaemolyticus* (1) and *V. neptunius* (1). Four of the isolates were not confirmed as vibrios: *Proteus mirabilis* (3) and *Enterococcus faecalis* (1) (Table 3).

The isolates not confirmed as members of the *Vibrio* genus were identified phenotypically by the A+/L–/O+ (Strain 42) combination, which points only to the species *V. coralliilyticus*, and A+/L+/O+ (Strains 7, 13, 22), which would point to 2 species *V. coralliilyticus* and *V. mimicus*. Larger imprecisions happened in combinations that point to more restrictive groupings, with less differential biochemical test results. Under this last combination fall most strains later identified as *V. alginolyticus*, *V. cholerae* and *V. neptunius*, which do not fit this profile. Amaral et al. (2014) have found genes in certain *Vibrio* species involved in metabolic routes related to ornithine decarboxylase, arginine dehydrolase, indole production and fermentation of galactose and other saccharides. In the literature, these strains are reported to not have metabolic activities related to these genes (i.e. A–/L–/O–): this false characteristic is used for phenotypic grouping in the identification keys.

Table 3. Comparison between the identification approaches to suspected *Vibrio coralliilyticus* strains

| Identification of isolates by phenotypic dichotomous key | Strain code(s) | Identification of isolates by sequencing |
|---|---|---|
| *V. coralliilyticus* | 15, 29, 32, 47, 49, 57, 62, 63, 70, 74, 75, 76, 77 | *V. alginolyticus* (13) |
| | 31, 71 | *V. cholerae* (2) |
| | 41 | *V. parahaemolyticus* (1) |
| | 26 | *V. neptunius* (1) |
| | 7, 13, 22 | *Proteus mirabilis* (3) |
| | 42 | *Enterococcus faecalis* (1) |

## CONCLUSION

The phenotypical characteristics considered for the differentiation between the species *V. coralliilyticus* and others are not efficient according to the results of the present study. More information about the diversity of the morphology of colonies and enzymatic activities must be considered in the formulation of phenotypic keys for *V. coralliilyticus* and related species. In addition, PCR analysis should be performed using more than one target gene, in order to avoid false positives or negative results.

It is important to remember that all approaches used to identify bacteria have limitations. No single test methodology provides completely accurate results, whether phenotypic or genotypic.

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