Australian and New Zealand Standard Diagnostic Procedures (ANZSDP) for the identification of *Vibrionaceae* from Australian and New Zealand aquatic animals using phenotypic and molecular procedures

Version April 2020

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Summary

The *Vibrionaceae* is a large and complex group of marine bacteria that can have a significant impact on the health of aquatic animals. A range of pathogenicity is seen among the species but a consistent feature is the opportunistic basis of infection. Nearly all phases of farm production are affected from larval rearing to competent adult animals. Disease outbreaks may occur in disparate aquatic animal groups including marine mammals, finfish, crustaceans, molluscs and zooxanthellae of coral. Some strains, however, can act as probionts and have proved effective as a means of controlling disease caused by other species of *Vibrionaceae*.

Identification

Routine, high-volume identification is achieved by phenotyping using standardised tests. To accommodate the large number of taxa and the phenotypic diversity that exists intra-species, identification is only practicable using computer-assisted probabilistic methods. The use of molecular tools for identification is increasing with the availability of economic DNA sequencing technologies. Reliable polymerase chain reaction (PCR) tests are available for several species and are a useful means of rapid screening or confirmatory identification.

Status of Australia and New Zealand

The range of *Vibrionaceae* associated with aquatic animals in Australia and New Zealand is relatively small despite the diversity of habitats, geographic range and climatic variation. Major pathogens encountered are *Photobacterium damselae* ssp. *damselae*, *Vibrio anguillarum* and *V. harveyi*.

More unusual species isolated, are *V. scophthalmi* (Atlantic salmon), *V. nigripulchritudo* (tropical rock lobster), *V. penaeicida* (southern rock lobster), *V. tapetis* (southern rock lobster and Atlantic salmon) and *Ph. damselae* ssp. *piscicida* (southern blue fin tuna and yellowtail kingfish).

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Introduction

The *Vibrionaceae* is an important and ubiquitous group of bacteria in marine and estuarine environments (Urakawa and Rivera 2006). Characteristically they have a close association with aquatic animals either as symbionts, normal flora or as pathogens. The ability of the group to occupy a wide diversity of habitats means that the *Vibrionaceae* have direct and indirect effects on aquaculture either as pathogens that are the cause of major disease, low grade infections that erode productivity or beneficial effects as probionts like some strains of *Vibrio alginolyticus* or *V. mediterranei* (Austin et al., 1995, Huys et al., 2001).

To varying degrees, *Vibrionaceae* have an intimate relationship with all farmed marine species of aquatic animals from live feed inputs such as Artemia and rotifers, crustaceans, bivalve and univalve molluscs to fin fish. The myriad of types, the range of species and their diverse role in aquaculture make the *Vibrionaceae* an important but challenging group of bacteria.

Taxonomy

The *Vibrionaceae* comprise seven genera: *Aliivibrio*, *Echinimonas*, *Enterovibrio*, *Grimontia*, *Photobacterium*, *Salinivibrio* and *Vibrio*. The genus *Listonella* has also been proposed and although a validly published name, there is now little evidence to justify its status, and use of the name is declining (Austin et al. 1997). Related to the *Vibrionaceae* is the family *Moritellaceae* and includes *Moritella marina* and *M.viscosa*, species pathogenic for salmonids.

The taxonomy of the *Vibrionaceae* is based largely on phylogenetic analysis of small subunit ribosomal DNA, in particular, the 16S rDNA gene sequence, supported by fluorescent amplified fragment length polymorphism (FAFLP) data (Thompson et al. 2001, 2006). FAFLP has proved useful as a means of species delineation because of the high level of 16S rDNA sequence similarity that exists between many species of the *Vibrionaceae*. More recently however, multilocus sequence analysis (MLSA) is being used to define the taxonomic boundaries of the family (Sawabe et al. 2013). Using MLSA, 22 clades are recognised within the *Vibrionaceae* (Table 1) (Sawabe et al. 2013).

Table 1 Clades of the family *Vibrionaceae*

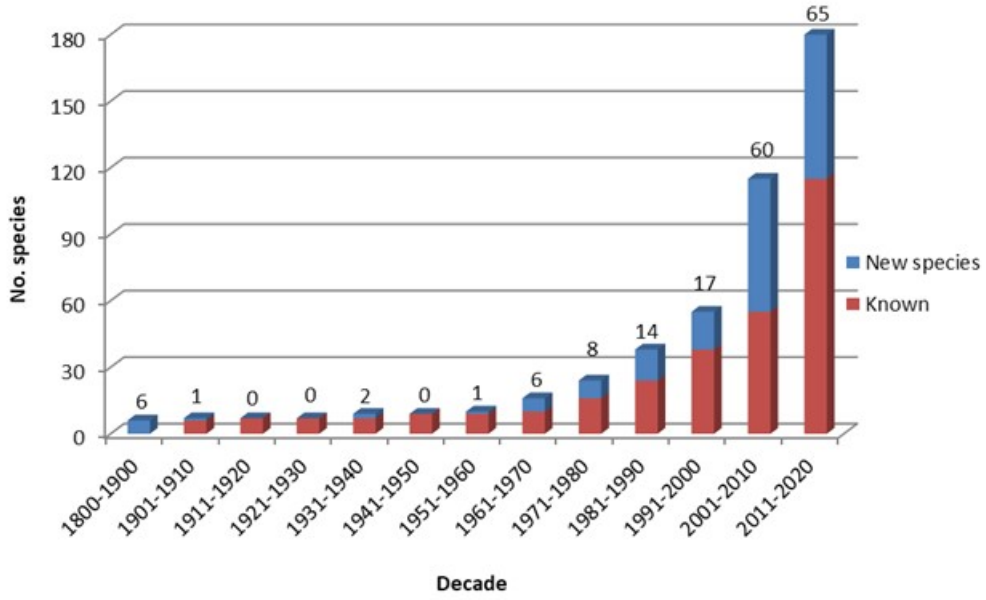
Clades A to H		
Anguillarum	Cholera	Coralliilyticus
Damselae	Diazotrophicus	Fischeri
Gazogenes	Halioticoli	Harveyi
Clades M to P		
Mediterranei	Nereis	Nigripulchritudo
Orientalis	Pectenicida	Phosphoreum
Porteresiae	Profundum	-

Clades R to V		
Rosenbergii	Rumoiensis	Scophthalmi
Splendidus	Vulnificus	-

Currently (2018), some 173 species across the seven genera of the *Vibrionaceae* are recognised, with a further seven species in the genus *Moritella*. Of the 180 species in total, 123 occur in the genus *Vibrio*. It should be borne in mind that the rate of discovery of new species over the past decade has increased markedly (Figure 1), which in itself adds particular challenges when considering a cohesive approach to the identification of species.

Phenotypes, when based on numerical taxonomy, are generally useful and predictive (Bryant et al. 1986, Carson et al. 2006), but where kit systems have been used to define species, invariably descriptions are poor and insufficient for the purpose of identification. There is marked genomic plasticity within the *Vibrionaceae*, which is seen as pronounced phenotypic diversity between and within species (Thompson et al 2004, Vandenberghe et al. 2003). As a consequence, finding traits that differentiate species continues to be a problem, particularly for those delineated by molecular taxonomy and where scant attention has been given to differential phenotyping.

Figure 1 Rate of increase in the discovery of species of the *Vibrionaceae*



Note: column data are the number of new species described per decade. Data obtained from the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.net/).

Diseases in aquatic animals

Disease involving *Vibrionaceae* is characterised by the opportunistic basis of infection. The pathogen may have an intimate association with the host as normal flora and may exhibit its pathogenic capacity if host defences are breached. This may occur either from stress events that lead to immunosuppression, physical damage to the integument, or the emergence of aggressive biovars within a population of aquatic animals. Severity of disease may range from fulminating septicaemias, typical of disease outbreaks, through to chronic infections that affect just a few individuals.

Of the 180 known species of *Vibrionaceae* and *Moritellaceae*, 90 species are found in association with aquatic animals. Of these species, 38 of them have been reported to be pathogenic or associated with diseased animals (Table 2). An awareness of them as potential pathogens is important.

Table 2 Species of *Vibrionaceae* as a cause of disease or associated with disease in aquatic animals

Vibrio species a to e		
Vibrio aestuarianus	*Vibrio alginolyticus*	*Vibrio anguillarum*
Vibrio bivalvicida	*Vibrio celticus*	*Vibrio cholerae*
Vibrio crassostreae	*Vibrio europaeus*	-
Vibrio species f to l		
Vibrio fluvialis	*Vibrio fortis*	*Vibrio furnissii*
Vibrio gigantis	*Vibrio harveyi*	*Vibrio ichthyoenteri*
Vibrio jasicida	*Vibrio kanaloae*	*Vibrio lentus*
Vibrio species m to p		
Vibrio mimicus	*Vibrio neptunius*	*Vibrio nigripulchritudo*
Vibrio ordalii	*Vibrio ostreicida*	*Vibrio owensii*
Vibrio parahaemolyticus	*Vibrio pectenicida*	*Vibrio penaeicida*
Vibrio proteolyticus	-	-
Vibrio species s to v		
Vibrio scophthalmi	*Vibrio splendidus*	*Vibrio tapetis*
Vibrio tubiashii	*Vibrio vulnificus*	-
Other species		
Aliivibrio wodanis	*Aliivibrio salmonicidae*	*Moritella marina*
Moritella viscosa	*Photobacterium damselae* ssp. *damselae*	*Photobacterium damselae* ssp. *piscicida*
Photobacterium toruni	-	-

Species of *Vibrionaceae* have an obligate requirement for sodium chloride (NaCl) (Goudie and Gow 1995) that ranges from as little as 5 to 15 mM for *V. cholerae* and *V. metschnikovii,* to 300 to 400 mM for *V. splendidus* (Baumann et al. 1980, 1984). A critical factor for potential pathogens is the coincidence of physiologies between host and pathogen. For osmoregulators such as fin fish, pathogens must have an optimum NaCl requirement close to physiological levels of vertebrates while for osmoconformers such as molluscs and crustaceans, pathogens have optimum NaCl concentrations close to that of seawater. Vibrios also have an absolute requirement for iron which is

of variable availability in the marine environment. It is likely that systemic infections are associated with iron availability as has been shown in human medicine. Extracellular pathogenicity factors such as siderophores, haemolysins, cytotoxins and proteases have been described in several species of *Vibrionaceae* and appear to be important in expression of disease (Allam et al. 2002, Liu and Lee 1999, Zhang eand Austin 2000).

Host range

Development of an exhaustive list of Australian and New Zealand host species serves little purpose. More importantly, cognisance of the range of host types is central to obtaining an understanding of the pathogenic versatility of the *Vibrionaceae*. In nearly every group of marine species, examples can be found of *Vibrionaceae* acting as pathogens. Major groups affected are: marine mammals, teleost fish, crustaceans, molluscs, both univalve and bivalve, and algae represented by the zooxanthellae of coral (Ben-Haim et al. 2003, Thompson et al. 2005, Sussman et al, 2008).

Species enzootic to Australia and New Zealand

Table 3 lists all of the *Vibrionaceae* known to occur in Australia and New Zealand and provides a guide to species that have been found associated with aquatic animals. Not all the species listed were found as pathogens.

Table 3 *Vibrionaceae* enzootic in Australia and New Zealand

Vibrio species a to f		
Vibrio alginolyticus	*Vibrio anguillarum*	*Vibrio chagasii*
Vibrio cholerae non-O1	*Vibrio cyclitrophicus*	*Vibrio diazotrophicus*
Vibrio furnissii	*Vibrio fluvialis*	-
Vibrio species h to l		
Vibrio halioticoli	*Vibrio harveyi*	*Vibrio ichthyoenteri*
Vibrio jasicida	*Vibrio lentus*	-
Vibrio species m to p		
Vibrio mediterranei	*Vibrio metschnikovii*	*Vibrio mimicus*
Vibrio mytili	*Vibrio natriegens*	*Vibrio navarrensis*
Vibrio nigripulchritudo	*Vibrio nereis*	*Vibrio orientalis*
Vibrio owensii	*Vibrio parahaemolyticus*	*Vibrio pelagius*
Vibrio penaeicida	*Vibrio proteolyticus*	-
Vibrio species s to v		
Vibrio scophthalmi	*Vibrio splendidus* biovar I	*Vibrio tapetis*
Vibrio tasmaniensis	*Vibrio tubiashii*	*Vibrio vulnificus* biovar I

Other species		
Aliivibrio fischeri	*Photobacterium damselae ssp. damselae*	*Photobacterium damselae ssp. piscicida*
Photobacterium iliopiscarium	-	-

Species exotic to Australia and New Zealand

Several pathogens have not been detected in association with aquatic animals and are considered exotic. These pathogens are listed in Table 4.

Table 4 *Vibrionaceae* exotic to Australia and New Zealand

Species	**Host**
Aliivibrio salmonicida	salmonids
Aliivibrio wodanis[a]	salmonids
Moritella viscosa	salmonids
Vibrio aestuarianus[b]	bivalve molluscs
Vibrio ordalii[a]	salmonids
Vibrio pectenicida	scallops
Vibrio crassostreae[a]	oysters
Vibrio ostreicida	oysters
Vibrio bivalvicida	clams
Vibrio celticus	clams
Vibrio europaeus	bivalve molluscs
Vibrio vulnificus biovar II	eels

Note: **a** has been detected in salmonids in New Zealand, **b** has been detected in molluscs in New Zealand.

Zoonotic agents

Some species of *Vibrionaceae* are the cause of zoonoses (Table 5) (Daniels and Shafaie, 2000). Infection invariably is the result of physical trauma arising from puncture wounds or the result of ingesting uncooked or improperly prepared seafood. High mortality and morbidity have been reported in the immunocompromised.

Table 5 Zoonotic *Vibrionaceae*

Vibrio species a to h		
Vibrio alginolyticus	*Vibrio cholerae*	*Vibrio cidicii*[a]

Vibrio species a to h		
Vibrio cincinnatiensis	*Vibrio fluvialis*	*Vibrio furnissii*
Vibrio harveyi	-	-
Vibrio species m to v		
Vibrio metschnikovii	*Vibrio mimicus*	*Vibrio navarrensis*[a]
Vibrio parahaemolyticus	*Vibrio vulnificus*	-
Other species		
Grimontia hollisae	*Photobacterium damselae ssp. damselae*	-

Note: **a** refer to Orata et al. 2016.

Characteristics of the *Vibrionaceae*

Sodium chloride is critical for the growth of *Vibrionaceae*. Many species have an obligate requirement for sodium ions and growth of nearly all species is stimulated by NaCl, even those that have a low requirement for NaCl, such as *V. cholera* (Singleton et al. 1982). Species of the *Vibrionaceae* do not in general have fastidious growth requirements and can be readily grown on peptone-based media as long as NaCl requirements are met. Growth of *Aliivibrio salmonicida*, *A. wodanis* and *M. viscosa*, is more reliable on media enriched with blood. In defined media some *Vibrionaceae* require supplementation with vitamins (Baumann et al. 1971), while most strains, even in complex media, respond well to the addition of low levels of yeast extract (Smith et al. 1991). As the natural habitat of the *Vibrionaceae* is the marine environment, better growth is obtained at a slightly alkaline pH in the range of 7.5 to 7.8. Many species and strains will form distinctive curved rods but this characteristic is not diagnostic of the *Vibrionaceae*. In tissue smears, rods can appear preternaturally large or pleomorphic but on culture will assume more typical form and proportions.

Uniformly, the *Vibrionaceae* are facultative anaerobes that ferment glucose. All species are oxidase positive with the exception of *V. metschnikovii*, which is oxidase negative. Species are sensitive to the vibriostat 0/129, a pteridine derivative related to trimethoprim (Matsushita at el. 1984). Some species however, such as *V. lentus*, may appear resistant if inappropriate test media are used or if strains have acquired resistance to trimethoprim from the drfA1 gene in plasmid class I integrons (Thungapthra et al. 2002). Most species of *Vibrionaceae* will grow at 25°C except for *A salmonicida*, *A. wodanis*, *M. marina*, *M. viscosa*, *Ph. iliopiscarium* and *Ph. phosphoreum*, which grow at 15°C. All zoonotic species will grow at 35 to 37°C.

Isolation strategies

Due to the widespread presence of *Vibrionaceae* species in marine and estuarine environments, particular care should be taken during isolation to ensure that isolates are of significance to the disease event. Wherever possible, samples should be collected using aseptic techniques from internal sterile sites, or from the internal surface of lesions rather than the external surface which may be heavily colonised by secondary microorganisms.

For osmoregulators, cultures from internal sites should be made on media enriched with blood such as blood agar base no. 2 (Oxoid) with 7% defibrinated sheep's blood as a non-selective medium

(Appendix A), and thiosulphate citrate bile sucrose (TCBS; Oxoid) agar as a selective indicator medium for *Vibrionaceae* (Baumann et al 1971). TCBS should be used only in conjunction with a non-selective medium as not all species will grow on TCBS.

For external sites on osmoregulators or any site of an osmoconformer, samples should be plated on either ZoBell's 2216E (ZMA) (Oppenheimer and ZoBell 1952) or Johnson's marine agar (JMA) (Johnson 1968) (Appendix A,) as non-selective media, and TCBS as the selective medium

For salmonids, particularly during periods of low water temperatures, samples from external sites should be plated on blood agar supplemented with 1.5% NaCl (Lunder et al. 1995). Plates should be incubated at 25°C for 2 to 3 days or at 15°C for up to 7 days for psychrophiles. Procedures for sampling should follow the ANZSDP guidelines for sample collection from finfish (Handlinger 2008).

Preservation

Cultures of *Vibrionaceae* can be held frozen at –80°C or in liquid nitrogen. A cryopreservative based on peptone and glycerol is suitable (Ward and Watt 1971) (Appendix A). Long-term storage based on freeze-drying is effective but the menstruum must be based on meso inositol (Redway and Lapage 1974) so as to regulate membrane phase transition temperature effects and protein stabilisation (Lunder et al. 1995) during freeze-drying and rehydration. Some species, notably *A. salmonicida* and *M. marina* have proved refractory to freeze-drying and are best preserved frozen.

Generally, cultures recover well from preservation. ZMA or JMA are suitable recovery media, but for more fastidious species, particularly *A. salmonicida*, *M. marina* and *M. viscosa*, blood agar supplemented with 2% sodium chloride (NaCl) should be used. A prudent strategy for recovery from freeze-drying is to use Vibrio Recovery Medium (Appendix A), which contains sodium pyruvate (Brewer et al. 1977, Mizunoe et al. 2000), which has been found useful in repair of damaged cells.

Identification strategies

Phenotyping

Typically, the *Vibrionaceae* exhibit a wide diversity of phenotype both between and within species. This heterogeneity in phenotype means that identification using a small number of tests either with keys or tables is unreliable. A more dependable strategy is to use a simultaneous polythetic approach combined with computer-assisted probabilistic identification (Leslie et al. 1995, Sneath 1974).

An identification matrix, VibEx7 (Appendix C), has been developed for species of *Vibrionaceae* associated with a diversity of aquatic animals in Australia from both temperate and tropical regions (Carson et al. 2006). The matrix can identify 63 species and biovars, and a further 25 as yet un-named protospecies of *Vibrionaceae* from aquatic animals. The panel of tests consists of 39 biochemical and 5 antibiotic sensitivity tests; details of the tests and formulations are given in Appendix B. The biochemical tests are in conventional tube or plate format. Alternatively, the panel of tests is available commercially in miniaturised format as MicroSys® V36 (DPIPWE, Launceston, Tasmania).

Probabilistic identification is undertaken using an implementation of Bayes Theorem (Wilcox et al. 1973). An identification is reached if the Willcox probability value P≥0.99 and the modal likelihood score is ≥0.001. The Willcox probability is a measure of the most likely identification, while the modal likelihood is a measure of the goodness-of-fit of the unknown to the nominated species (Gyllenberg and Niemlä 1975). Probabilistic identification is undertaken using PIBWin (Bryant 2004), an intuitive software package for a Microsoft Windows operating system. The application is available from the Centre for Aquatic Animal & Vaccines, DPIPWE, Launceston, Tasmania.

If VibEx7 is not available, conventional biochemical tests and API 20E kits may be used with identification charts in Buller (2014).

MALDI-TOF

MALDI-TOF: Matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry is becoming more widely available. The current MALDI BioTyper (Bruker Daltonics) database consists of almost 8,000 organisms and contains 106 strains covering 52 of the 147 described Vibrio species; 3 strains covering 2 species of the 33 described Photobacterium species, and 1 species of the 6 described Aliivibrio species.

Molecular

Identification of *Vibrionaceae* using molecular methods is controversial. Amplified fragment length polymorphism (AFLP) and multilocus sequence analysis (MLSA) (Thompson et al. 2005) have proved important in establishing the taxonomic structure of the *Vibrionaceae* but these procedures are not yet suitable as a means of high-volume routine identification purposes. For isolates that are of disease significance, which are unable to be identified readily by other means, MLSA should be used. Allocation of an unknown or unidentified isolate to one of the known 22 clades of *Vibrionaceae*, can be accomplished using a panel of 8 housekeeping genes: 16S rRNA gene, *gapA*, *gyrB*, *ftsZ*, *mreB*, *pyrH*, *recA*, *rpoA* and *topA* (Sawabe et al. 2013).

PCR amplification of sequence motifs characteristic of species is a practical means of molecular identification but the scope of application is limited (Nishibuchi 2006). The most widely described and conserved construct is the 16S rRNA gene, but for the *Vibrionaceae* (Ruimy et al., 1994) sequence divergence is only 9%, which greatly limits the possibilities of identifying motifs that are species unique. Sequences other than 16S rRNA have been identified, typically virulence factors, but the constancy of these targets across strains of a species is unknown and their suitability as constructs for identification purposes is questionable. Alternatively, primers targeting housekeeping *atpA* gene have shown greater discriminatory power and better intra-species variation than those targeting the 16S rRNA (Thompson et al 2007).

Species specific recommended PCR primer sets relevant for *Vibrionaceae* of aquatic animals are for *Ph. damselae* and *V. harveyi*. Based on the 16S rRNA gene both primer sets are suitable for species identification. The PCR for *P. damselae* is in multiplex format (Osorio et al. 2000) with one primer pair specific for *Ph. damselae*, *sensu lato*, and a second primer pair for the urease gene *ureC* that is specific for *Ph. damselae* ssp. *damselae*. Evidence of *Ph. damselae* ssp *piscicida* is inferred by the absence of a *ureC* amplicon. The primer pair for *V. harveyi* (Oakey et al 2003) is compromised to some extent by known cross-reaction with some, but not all, strains of *V. alginolyticus*. For the primers to be truly discriminating, positive PCR reactions must be verified using at least one of the phenotypic tests listed in Table 6.

Table 6 Differential phenotypic tests for *Vibrio harveyi* and *Vibrio alginolyticus*

		Test	
Species	**PNPG[a]**	**Aesculin hydrolysis**	**Utilisation of putrescine**
Vibrio alginolyticus	16%	10%	100%
Vibrio harveyi biovar I	95%	95%	2%
Vibrio harveyi biovar II	100%	100%	0%

Note: tests are for confirming a positive PCR reaction for 16S rRNA *V. harveyi* primers. **a** 2-nitrophenyl β-D-galactopyranoside.

Identification by PCR should be limited to pure cultures and used as a means of confirming the identity of strains with atypical phenotypes. Performance of the primers has not been validated for the purpose of direct detection in tissues or environmental samples.

A range of primer sets for other constructs in the *Vibrionaceae* have been described, of which some have been critically evaluated. Of these, primers for the *cth* cytolysin/haemolysin gene of *V. vulnificus* (Brasher et al. 1998) and the *vah1* haemolysin gene of *V. anguillarum* (Osorio et al. 2000) appear robust.

Use of these primers for identification in the absence of other species defining characteristics is not recommended since the frequency at which the targets occur intra-species is not known. The primers may have value however for screening purposes, particularly for strains where the target is known to occur or for establishing the presence of virulence factors.

Quality control

The most important factor determining success in identification is the use of standardised tests. Tests of different format should not be used unless extensive testing has been undertaken to verify test equivalence. It is important to recognise and identify sources of error that, if not well controlled, can result in unreliable identification outcomes (Sneath 1974).

Intrinsic error is associated with some tests and some species that can result in variable test outcomes. A second form of error arises from procedural deficiencies, particularly interpretation of weak positive tests.

Regular use of quality control organisms is recommended (Table 7), together with trend analysis to identify drift in performance. With practice, intra-laboratory test error of 2% is achievable (Carson et al. 2006, Lapage et al. 1973, Sneath and Johnson 1972).

Table 7 *Vibrio* quality control strains

Species	Strain
Vibrio anguillarum	ATCC 19264^T
Vibrio fluvialis	NCTC 11327^T
Vibrio mediterranei	CIP 103203^T
Vibrio parahaemolyticus	ATCC 17802^T
Vibrio tubiashii	NCIMB 1340^T

Limitations

The identification matrix VibEx7 reflects the diversity of *Vibrionaceae* associated with aquatic animals in Australia. It does not however include all the validly published species of *Vibrionaceae* as listed in Table 8 and these species will not be identified using the matrix. It should be noted however, that identification by phenotype can be achieved by reference to the phenotypes published for these species using the tests described in section 2 test methods.

Data for new species as they occur can be readily added to the VibEx7 matrix. The ability to differentiate the new species from those already in the matrix can be assessed using the IDSC tool in the PIBWin software.

Table 8 Species of *Vibrionaceae* associated with animals

Vibrio species a to e		
Vibrio algivorus	Vibrio artabrorum	*Vibrio atlanticus*
Vibrio atypicus	*Vibrio barjaei*	*Vibrio bivalvicida*
Vibrio brasiliensi	*Vibrio breoganii*	*Vibrio caribbeanicus*
Vibrio celticus	*Vibrio comitans*	*Vibrio cortegadensis*
Vibrio crassostreae	*Vibrio crosai*	*Vibrio europaeus*
Vibrio ezurae	-	-
Vibrio species f to k		
Vibrio fortis	*Vibrio galatheae*	*Vibrio gallaecicus*
Vibrio gallicus	*Vibrio gigantis*	*Vibrio hemicentroti*
Vibrio hepatarius	*Vibrio hippocampi*	*Vibrio hispanicus*
Vibrio inusitatus	*Vibrio kanaloae*	-
Vibrio species m to p		
Vibrio maritimus	*Vibrio mexicanus*	*Vibrio neonatus*
Vibrio neptunius	*Vibrio ostreicida*	*Vibrio panuliri*
Vibrio pomeroyi	*Vibrio ponticus*	-
Vibrio species r to x		
Vibrio rarus	*Vibrio renipiscarius*	*Vibrio rotiferianus*
Vibrio sinaloensis	*Vibrio sonorensis*	*Vibrio stylophorae*
Vibrio superstes	*Vibrio variabilis*	*Vibrio xuii*
Other species		
Aliivibrio finisterrensis	*Echinimonas agarilytica*	*Enterovibrio norvegicus*
Photobacterium aplysiae	*Photobacterium jeanii*	*Photobacterium kishitanii*
Photobacterium panuliri	*Photobacterium sanguinicancri*	*Photobacterium swingsii*
Photobacterium toruni	-	-

Note: some of species listed are pathogens. Listed species are not included in the VibEx7 identification matrix.

Identification by MALDI-TOF is limited by the commercially supplied database, however the MALDI-BioTyper (Bruker Daltonics) allows users to build a custom database. Phenotyping by conventional

biochemical identification, although limited by compiled reference material, does allow comparison to the original published description of the bacterium.

2. Test methods

Approaches to the identification of the *Vibrionaceae*

Identification of species of the *Vibrionaceae* is challenging given the diversity and number of species involved. Consideration of a suitable approach needs to take in to account several factors, not least of which is the utility of a selected approach, purpose, turnaround time in a diagnostic context, volume of samples, cost and available technical expertise.

The approaches to identification of the *Vibrionaceae* are summarised in Table 9. Phenotyping and MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) are well suited to high volume throughput but are dependent on the quality of the accompanying reference databases required for reaching an identification. PCR has value where detection of a known species is required but has little value for prospective work, that is, where identification of known unknowns occurs, typical of diagnostic work. Genome sequencing represents a gold standard but technologies are not yet sufficiently developed for economical, high-throughput application with rapid turnaround times.

Table 9 Advantages and disadvantages of identification strategies

Approach	Advantages	Disadvantages
Phenotyping	• Inexpensive • Fast • Useful for strain detection • Convenient in miniaturised format • Suitable for high volume throughput.	• Dependent on database quality • Cumbersome with macro-format tests • Test manufacture time consuming • Plasticity of phenotypes.
MALDI-TOF	• Fast • Inexpensive running cost • Sample preparation straightforward • Suitable for high volume throughput.	• High capital cost of equipment • Dependent on database quality.
PCR identification	• Fast • Good retrospectivity • High volume throughput.	• Limited to detection of what is known • Multiplexing for multiple becomes cumbersome.
Genomic sequencing	• MLSA a reference standard • Good prospectivity • Ideal for unusual or hard to identify isolates in high value situations.	• Expensive reagent and analysis costs • Time consuming processing and analysis of isolates • Data analysis complex • Not suited to high volume processing • Dependent on database quality.

Detailed in this ANZSDP is an approach to phenotyping using probabilistic identification and description of PCR procedures for selected pathogens. An approach is provided for multi-locus sequence analysis (MLSA) of isolates where a definitive identification is required for biosecurity investigations or identification of a novel pathogen. Ultimately, the approach used for identification must meet the practicalities and needs for a given situation.

Identification by phenotype

Principle

Identification of *Vibrionaceae* is made using the entire panel of tests listed in Table 10 using the standardised test formulations given in Appendix B. An identification is made by matching the phenotype of an unknown against the probability data matrix VibEx7 and determining the most likely identification using the computer software package PIBWin.

Table 10 Panel of tests for the identification of *Vibrionaceae*

Test	**Types (where applicable)**
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Acid (fermentation)	• Arbutin • Mannitol • Salicin • Sucrose • Gentiobiose.
Growth	• 7% NaCl • 10% NaCl.
Acetoin	-
Indole	-
Alkaline phosphatase, pH 8.0	-
Oxidase	-
Hydrolysis	• 2-nitrophenyl β-D-galactopyranoside • L-glutamic acid 5-(4-nitroanilide) • 4-nitrophenyl sulphate • Aesculin • Agar • Gelatin • Starch.
Sole carbon utilisation	• α-ketoglutarate • Acetate • D-alanine • Citrate • L-citrulline • D-galactose • D-gluconate • D-glucosamine

Test	Types (where applicable)
	• D-glucose • D-glucuronate • Glycerol • L-histidine • DL-3-hydroxybutyrate • trans-4-hydroxy-L-proline • DL-lactate • D-lactose • Propionate • Putrescine • Succinate • Sucrose.
Resistance	• 0/129 10 µg • 0/129 150 µg • Ampicillin 10 µg • Carbenicillin 100 µg • Novobiocin 5 µg.

Sample requirements

Pure cultures, less than 48 hours old, should be used for the inoculum. Cultures recovered from preservation by freezing or drying must be subcultured at least twice before commencing identification.

Test procedure

Perform an oxidase test by a preferred method. Observe agarolytic activity as pitting of colonies on maintenance agar such as JMA or ZMA (Table 31 and Table 34).

Inocula

Prepare two inocula in 3 mL volumes of 2% saline: one to a density equal to McFarland 0.5, the other to McFarland 2.

Inocuation of media

Inoculate the decarboxylase test media with 100 µL of McFarland 2 density cell suspension. Inoculate the remaining liquid media with 100 µL of McFarland 0.5 density cell suspension. For the acetoin and arginine dihydrolase tests, inoculate the semi solid media with a straight wire.

Sole carbon source media are spot inoculated with 2 µL of McFarland 0.5 suspension or with a multipoint inoculator. Maximum number of inocula on a plate should not exceed 30.

Gelatin and starch plates are spot inoculated with 2 µL of McFarland 0.5 suspension or with a multipoint inoculator. Maximum number of inocula on a plate should not exceed 6, well separated inoculum points.

Tests for arginine dihydrolase and decarboxylases are overlayed with 20 to 25 mm of sterile liquid paraffin.

Sensitivity tests are undertaken on Mueller-Hinton agar supplemented with 2% w/v NaCl. The medium is inoculated with the McFarland 0.5 suspension.

Incubation

Tests are incubated at 25°C for 48 hours. Observe tests daily and record changes. Sensitivity tests are incubated for 24 hours and the diameter of the zone of inhibition measured.

Psychrophilic species are tested at 15°C for 8 days; sensitivity tests for 3 to 4 days. Known psychrophiles are:

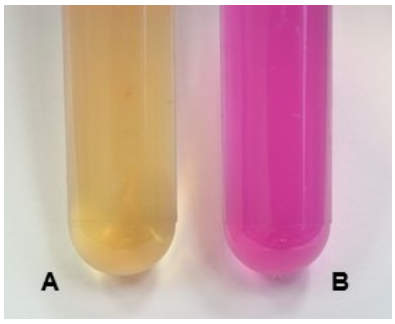
- *Aliivibrio logei*
- *Aliivibrio wodanis*
- *Aliivibrio salmonicida*
- *Moritella marina*
- *Moritella viscosa*
- *Photobacterium iliopiscarium*
- *Photobacterium phosphoreum.*

Test interpretation

Arginine dihydrolase test

For the arginine dihydrolase test a positive reaction is a pink to red colour and a negative reaction is yellow to orange (Figure 2).

Figure 2 Arginine dihydrolase test



Note: **A** negative reaction, **B** positive reaction.

Decarboxylase tests

For the decarboxylase test the negative control should be yellow for the test to be valid. A positive test for ornithine or lysine decarboxylase is purple (Figure 3).

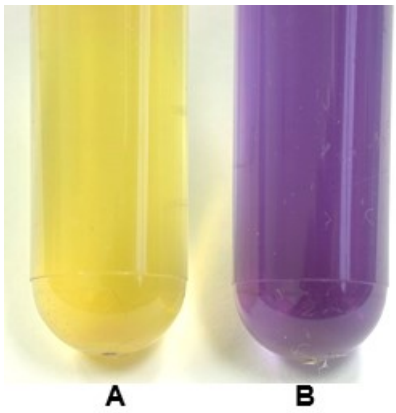
Figure 3 Decarboxylase tests

Note: **A** positive reaction for orthithine, **B** positive reaction for lysine, **C** negative control reaction.

Acid from carbohydrates

For the acid fermentation test a dirty yellow to bright yellow colour is positive and a pale purple to deep purple is negative (Figure 4). Protein deamination may occur with prolonged incubation and may cause positive tests to appear negative due to alkaline pH shifts; ignore reversions.

Figure 4 Acid fermentation test



Note: **A** positive reaction, **B** negative reaction.

Growth

For NaCl tolerance tests any sign of growth is a positive reaction.

Acetoin (Voges-Proskauer) test

For the acetoin test overlay semi-solid medium with 200 µL of α-naphthol followed by 100 µL of KOH or creatine. Leave at room temperature for up to 30 minutes. A pale pink to red layer is positive and a yellow to tan layer is negative (Figure 5).

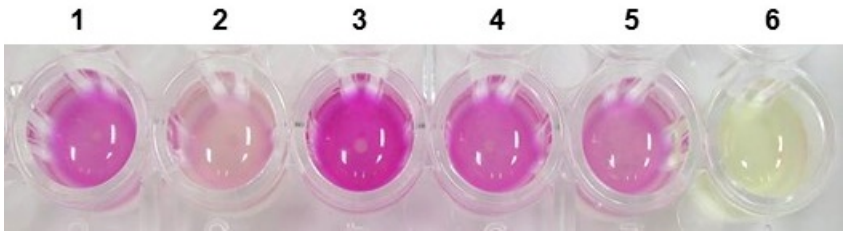
Figure 5 Acetoin test

Note: **A** positive reaction, **B** negative reaction.

Indole test

In a microtitre tray well add 100 µL of Kovács' indole reagent to an equal volume of culture. Mix the contents of the well by careful aspiration with a pipette. If a pink to red colour is visible record as positive (Figure 6). If in doubt, remove contents with a glass Pasteur pipette and allow the phases to separate in the pipette body; record the reaction based on the top phase only.

Figure 6 Indole test



Note: Wells 1 to 5 show a range of positive reactions. Well 6 is a negative reaction.

Gelatin hydrolysis

For a gelatin hydrolysis test flood the plate with saturated ammonium sulphate. Any zone of clearing around the inocula is a positive reaction.

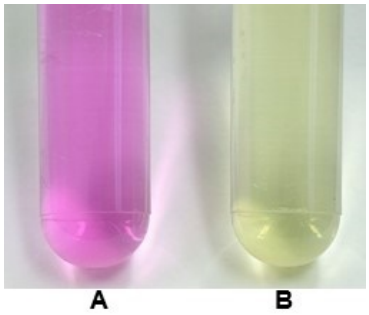
Starch hydrolysis

For a starch hydrolysis test flood the plate with Gram's iodine. Zones of yellow to light tan colour around the inocula is positive, while black to dark blue is negative.

Urease

For a urease test, a pink to red colour indicates a positive reaction. A yellow reaction is negative (Figure 7).

Figure 7 Urease test

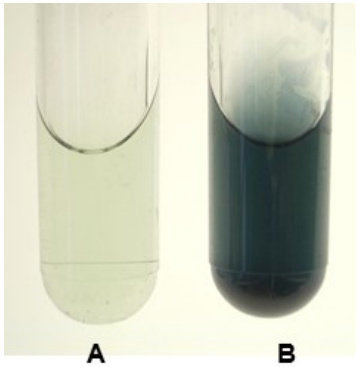


Note: **A** positive reaction, **B** negative reaction.

Chromogens

For a indoxyl alkaline phosphatase test any blue to black colour is positive and no colour is negative (Figure 8).

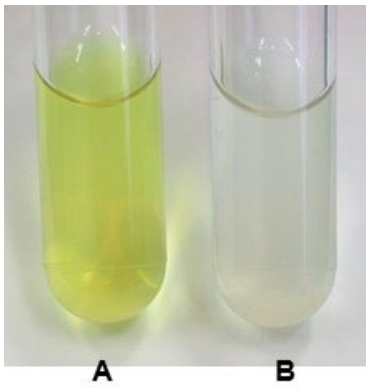
Figure 8 Indoxyl phosphate for alkaline phosphatase test



Note: **A** negative reaction, **B** positive reaction.

For a nitrophenol & nitroaniline chromogens test any yellow colour is positive and no colour is negative (Figure 9).

Figure 9 nitrophenol & nitroaniline chromogens test

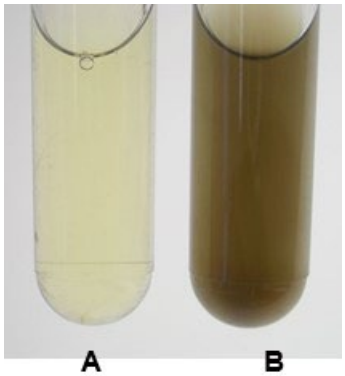


Note: Substrate is 2-nitrophenyl β-D-galactopyranoside (PNPG). **A** positive reaction, **B** negative reaction.

Aesculin test

For an aesculin test a brown to black colour is positive for hydrolysis and a very light tan to colourless is negative (Figure 10).

Figure 10 Aesculin test

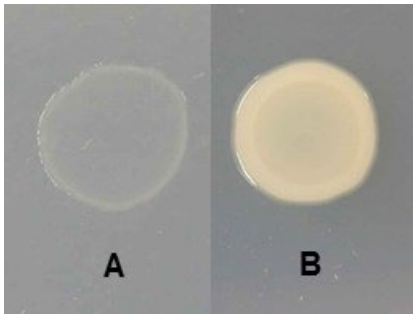


Note: **A** negative reaction, **B** positive reaction

Sole carbon source tests

For a sole carbon source test examine the negative control plate; some strains and some species may show very slight growth even when using purified agar (Figure 11).

Figure 11 Sole carbon source test



Note: **A** negative control, **B** glucose. Test organism is *Vibrio fluvialis* NCTC 11 327T. Differentiation between positive and negative tests.

With reference to the control plate, examine growth on the remaining test plates. Growth in excess of the negative control is read as positive. If there is no growth with glucose the strain may be nutritionally fastidious. Retest with sole carbon source media containing 0.015 g/L Casamino acids.

Antibiotic sensitivity test

For antibiotic sensitivity tests the diameter of the zone of inhibition is interpreted using the data in Table 11. The disc size is 6 mm.

Table 11 Zone size interpretation for diagnostic antibiotics

Test	Resistant	Sensitive
0/129 10 µg	≤15 mm	≥16 mm
0/129 150 µg	No zone	Any zone
Ampicillin 10 µg	≤13 mm	≥14 mm
Novobiocin 5 µg	≤16 mm	≥17 mm
Carbenicillin 100 µg	≤22 mm	≥23 mm

Probabilistic identification

An identification is obtained using the software PIBWin and the probability matrix VibEx7 (Appendix C). An identification is accepted if the Willcox probability score P is ≥0.99 and the modal likelihood score (MLS) is ≥0.001 (Carson et al. 2006).

If P is ≥0.99 but the MLS is <0.001, the unknown may represent an outlier of the nominated species. Differences between expected and observed reactions should be inspected to determine whether the suggested identification can be accepted. If test variance for unexpected results is between 30% and 70% then the identification can be accepted but should be reported as a poor fit that represents a species outlier. If observed and expected reactions for two or more tests are in complete disagreement then the suggested identification is erroneous and the unknown should be reported as unidentifiable.

Each species in the data matrix is proceeded by the number of strains used to establish the phenotypic range. For taxa based on only a few strains, an acceptable match will occur only if the unknown is very similar to the species description. Since the estimate of diversity is narrow, it is likely that strains of the species may appear as outliers.

In some instances, an unknown may appear to span two near related taxa such as biovars of a species. If the first and second most likely identifications are for related taxa then the probability values can be summed (Bascombe et al. 1973), and if Psum is ≥0.99, the unknown can be assigned to the level of taxon complex or group.

Identification of members of the Harveyi clade a two-step process of identification is used. The VibEx7 probability matrix allocates an unknown to the Harveyi clade. If an acceptable identification is reached, the phenotypic profile is then assessed against the clade-specific matrix for members of the Harveyi clade. Identification of the unknown is then made against this more closely defined matrix that contains only species of the Harveyi clade.

The profile for *Vibrio tapetis* is weighted to Australian isolates from rock lobster and salmonids. The phenotype is consistent with *V. tapetis* ssp. *britannicus* or *V. tapetis* ssp. *quintayensis* rather than *V. tapetis* ssp. *tapetis* associated with brown ring disease in clams. As the phenotype of *V. tapetis* ssp.

tapetis is under represented in the VibEx7 database, the modal likelihood score will be low. In such instances, confirmation of identity should be undertaken by MLSA.

Care must be exercised when accepting a computer-assisted identification. Ultimately, professional judgement and microbiological sense must be used for interpretation.

Identification by PCR

Introduction

The primer sets have been evaluated and optimised for the purpose of culture identification. The protocols represent optimal conditions but minor refinements may be required to account for variation in the characteristics of different thermal cyclers. Should *atpA* PCR be considered for the analysis of vibrios refer to Thompson et al. (2007).

Extraction of DNA

Reference DNA

Extract and purify DNA from control strains using a suitable commercial DNA extraction kit. Purified control DNA is used at a concentration of 50 pg μL^{-1}.

Sample DNA

To 100 µL of PCR grade 18MΩ water in a 1.5 mL microfuge tube, suspend sufficient cells to a density equivalent to McFarland 1. Hold the tube at 100°C in a dry-heat block for 15 minutes and then cool rapidly in a cool block for 5 minutes. Pellet the cells at 8,000 rcf for 10 minutes and collect the supernatant containing liberated DNA. The extracted DNA is suitable for amplification without purification.

PCR reaction volume

All PCR reactions are as 25 µL volumes in 200 µL thin-walled tubes.

Standard PCR reagents

Standard PCR reagents for the PCR primer sets are:

- PCR grade water, 18MΩ
- 50 mM magnesium chloride
- 16 mM dNTP stock (4mM each dNTP)
- A suitable high-fidelity Taq DNA polymerase
- Buffer for Taq DNA polymerase
- Primers (20 µM stock).

If a commercial master mix is used for PCR reactions in this document it must be verified in-house to ensure comparability.

Electrophoresis of amplicon

Amplicon is visualised by electrophoresis using 2% agarose gel containing 0.5 µg mL^{-1} ethidium bromide and 1 × TBE buffer (Table 12). Use a 100 bp ladder as a comparative index of amplicon size. Gels should be run at 7 volts cm^{-1} constant voltage.

Table 12 Ingredients for 10× Tris-Boric-EDTA buffer

Ingredient	Amount
Tris (base)	108.0 g
Boric acid	55.0 g
EDTA	8.3 g

Note: the buffer pH should be 8.0.

Photobacterium damselae PCR

(Osorio et al. 2000)

Targets

For the *Photobacterium damselae* PCR the 16S rRNA gene is used for species identification and the *ureC* gene for subspecies identification.

Primers and controls

The PCR primers used in the *Photobacterium damselae* PCR are summarised in Table 13. The DNA controls are summarised in Table 14.

Table 13 *Photobacterium damselae* PCR primers

Primer	Sequence
Car1	5'-GCT TGA AGA GAT TCG AGT-3'
Car2	5'-CAC CTC GCG GTC TTG CTG-3'
Ure-5'	5'-TCC GGA ATA GGT AAA GCG GG-3'
Ure-3'	5'-CTT GAA TAT CCA TCT CAT CTG C-3'

Table 14 *Photobacterium damselae* PCR DNA controls

DNA control
Ph. damselae ssp *damselae* NCIMB 2184T
Ph. damselae ssp *piscicida* NCIMB 2058T
Ph. iliopiscarium ATCC 51760T

Master mix and cycle parameters

The master mix used in the *Photobacterium damselae* PCR is summarised in Table 15. The cycle parameters are shown in Table 16.

Table 15 *Photobacterium damselae* PCR master mix

Component	Volume
Water	11.15 µL
50 mM $MgCl_2$	1 µL
10× reaction buffer	2.5 µL
16 mM dNTPs	1.25 µL
20 µM Car1	2 µL
20 µM Car2	2 µL
20 µM Ure-5'	2 µL
20 µM Ure-3'	2 µL
5 U μL^{-1} Taq polymerase	0.1 µL
Template DNA	1 µL
Total volume	25 µL

Table 16 *Photobacterium damselae* PCR cycle parameters

Temperature	Time
1 cycle	
95°C	4 minutes
30 cycles	
95°C	1 minute[a]
60°C	1 minute
72°C	40 seconds
Final extension	
72°C	5 minutes

Note: **a** denaturation cycle may need to be verified in-house.

Interpretation

Ph. damselae ssp *damselae*: species specific amplicon at 267 bp for 16S rDNA with an additional amplicon at 448 bp for the *ureC* gene that is diagnostic for the subspecies damselae.

Ph. damselae ssp *piscicida*: a single amplicon at 267 bp for 16S rDNA. Absence of an amplicon for *ureC* gene is diagnostic for the subspecies piscicida.

Limitations

A single band of 267 bp is diagnostic of *Ph. damselae sensu lato*. An identification of *Ph. damselae* ssp *piscicida* is inferred if only this band is present, but some caution needs to be used where an identification is reached on the basis of absence. Corroborating phenotypic evidence and complete 16S rRNA gene sequence should be obtained if the identification represents a new finding for a region or host not previously associated with *Ph. damselae* ssp *piscicida*.

Vibrio harveyi PCR

(Oakey et al. 2003)

Targets

The target of the *Vibrio harveyi* PCR is the 16S rRNA gene.

Primers and controls

The PCR primers used in the *Vibrio harveyi* PCR are summarised in Table 17. The DNA controls are summarised in Table 18.

Table 17 *Vibrio harveyi* PCR primers

Primer	Sequence
VH-1	5'-AAC GAG TTA TCT GAA CCT TC-3'
VH-2	5'-GCA GCT ATT AAC TAC ACT ACC-3'

Table 18 *Vibrio harveyi* PCR DNA controls

DNA control
V. harveyi ATCC 14126^T
V. alginolyticus ATCC 17749^T

Master mix and cycle parameters

The master mix used in the *Vibrio harveyi* PCR is summarised in Table 19. The cycle parameters are shown in Table 20.

Table 19 *Vibrio harveyi* PCR master mix

Component	Volume
Water	18.15 µL
50 mM $MgCl_2$	1.5 µL
10× reaction buffer	2.5 µL
16 mM dNTPs	1.25 µL
20 µM VH-1	0.25 µL
20 µM VH-2	0.25 µL

Component	Volume
5U μL^{-1} Taq polymerase	0.1 µL
Template DNA	1 µL
Total volume	25 µL

Table 20 *Vibrio harveyi* PCR cycle parameters

Temperature	Time
1 cycle	
94°C	2 minutes
40 cycles	
94°C	1 minute
65°C	1 minute
72°C	2 minutes
Final extension	
72°C	5 minutes

Interpretation

A single band of 413 bp is characteristic of *V. harveyi*.

Limitations

On the basis of testing undertaken, the primers are specific for strains of both *V. harveyi* biovar I and *V. harveyi* biovar II associated with blister disease in abalone. Some strains of *V. alginolyticus* are known to cross-react because of sequence similarity with the primer regions. A positive finding must be corroborated by phenotype using the tests given in Table 10.

Vibrio vulnificus PCR

(Brasher et al. 1998)

Targets

The target of the *Vibrio vulnificus* PCR is the *cth* cytolysin/haemolysin gene.

Primers and controls

The PCR primers used in the *Vibrio vulnificus* PCR are summarised in Table 21. The DNA controls are summarised in Table 22

Table 21 *Vibrio vulnificus* PCR primers

Primer	Sequence
L-CTH	5’-TTC CAA CTT CAA ACC GAA CTA TGA C-3’

Primer	Sequence
R-CTH	5’-GCT ACT TTC TAG CAT TTT CTC TGC-3’

Table 22 *Vibrio vulnificus* PCR DNA controls

DNA control
V. vulnificus ATCC 27562^{T}
V. parahaemolyticus ATCC 17802^{T}

Master mix and cycle parameters

The master mix used in the *Vibrio vulnificus* PCR is summarised in Table 23. The cycle parameters are shown in Table 24.

Table 23 *Vibrio vulnificus* PCR master mix

Component	Volume
Water	16.375 µL
50 mM $MgCl_2$	1.25 µL
10× reaction buffer	2.5 µL
16 mM dNTPs	1.25 µL
20 µM L-CTH	1.25 µL
20 µM R-CTH	1.25 µL
5U μL^{-1} Taq polymerase	0.125 µL
Template DNA	1 µL
Total volume	25 µL

Table 24 *Vibrio vulnificus* PCR cycle parameters

Temperature	Time
1 cycle	
94°C	3 minutes
40 cycles	
94°C	1 minute
57°C	1 minute

Temperature	Time
72°C	3 minutes
Final extension	
72°C	5 minutes

Interpretation

A single band of 205 bp is characteristic of the cytolysin/haemolysin gene in *V. vulnificus*.

Limitations

An identification of *V. vulnificus* is inferred by the presence of the *cth* gene which appears to be specific for *V. vulnificus*. It is noteworthy that the forward primer L-CTH is unique to *V. vulnificus* while R-CTH is homologous with the thermolabile haemolysin gene of *V parahaemolyticus* (Taniguchi et al. 1986). Based on limited testing the primers appear specific for *V. vulnificus,* however the frequency with which the gene occurs within the species is not known and may not be sufficiently reliable for the purpose of identification in the absence of a priori information.

Vibrio anguillarum PCR

(Hirono et al. 1996)

Targets

The target of the *Vibrio anguillarum* PCR is the *vah1* haemolysin gene.

Primers and controls

The PCR primers used in the *Vibrio anguillarum* PCR are summarised in Table 25.The DNA controls are summarised in Table 26.

Table 25 *Vibrio anguillarum* PCR primers

Primer	Sequence
VaH1-P1	5’-ACC GAT GCC ATC GCT CAA GA-3’
VAH1-P2	5’-GGA TAT TGA CCG AAG AGT CA-3’

Table 26 *Vibrio anguillarum* PCR DNA controls

DNA control
V. anguillarum ATCC 19264^{T}
V. parahaemolyticus ATCC 17802^{T}

Master mix and cycle parameters

The master mix used in the *Vibrio anguillarum* PCR is summarised in Table 27. The cycle parameters are shown in Table 28.

Table 27 *Vibrio anguillarum* PCR master mix

Component	Volume
Water	14.4 µL
50 mM MgCl2	0.75 µL
10× reaction buffer	2.5 µL
16 mM dNTPs	1.25 µL
20 µM VaH1-P1	2.5 µL
20 µM VaH1-P2	2.5 µL
5U μl^{-1} Taq polymerase	0.1 µL
Template DNA	1 µL
Total volume	25 µL

Table 28 *Vibrio anguillarum* PCR cycle parameters

Temperature	Time
1 cycle	
94°C	4 minutes
30 cycles	
94°C	30 seconds
55°C	30 seconds
72°C	1 minute
Final extension	
72°C	5 minutes

Interpretation

A single band of 490 bp is characteristic of the haemolysin gene of *V. anguillarum*.

Limitations

The *vah1* gene appears to be specific to *V. anguillarum* despite having a common ancestry with haemolysins from other species of *Vibrionaceae*. From limited testing however, it appears that not all strains of *V. anguillarum* possess the *vah1* gene and its use as a primary means of identification is limited. Primers for the *vah1* gene should be limited to determining the presence of the haemolysin gene in strains or as a supporting test.

Identification by multilocus sequence analysis

Introduction

The primer sets have been evaluated and optimised for the purpose of Multilocus Sequence Analysis (MLSA) for the phylogenetic analysis of members of the *Vibrionaceae*. The analysis may be for the purpose of identifying an unknown isolate or confirmation of an identification reached by other means such as phenotyping. The protocols represent optimal conditions but minor refinements may be required to account for variation in the characteristics of different reagents and thermal cyclers.

MLSA primer set

The primers required for MLSA for the *Vibrionaceae* are given in Table 29.

Table 29 MLSA primers for the phylogenetic analysis of the *Vibrionaceae*

Name	Forward	Reverse
gapA[a]	150F: 5'-ACT CAY GGY CGT TTC AAC GGY AC-3'	957R: 5'-RCC GAT TTC GTT RTC GTA CCA AG-3'
ftsZ[a]	150F: 5'-ACT CAY GGY CGT TTC AAC GGY AC-3'	800R: 5'-GCA CCA GCA AGA TCG ATA TC-3'
topA[a]	75F: 5'-GCT GTT GAA CAC ATG GTA CG-3'	1200R: 5'-GAA GGA CGA ATC GCT TCG TG-3'
mreB[a]	400F: 5'-GAG ATC ATC GGT GGT GAT G-3'	999R: 5'-CCG TGC ATA TCG ATC ATT TC-3'
gyrB[a]	360F: 5'-GTG GTT CTA CCC AAG TTG AG-3'	1001R: 5'-CCT TTA CGA CGA GTC ATT TC-3'
rpoA[a]	300F: 5'-TCA TCA TGA CGG TAC TGC AC-3'	500R: 5'-TTG GAC GCT CAT CTT CTT C-3'
pyrH[a]	1F: 5'-ATG CAG GGT TCT GTA ACA G-3'	530R: 5'-TAG GCA TTT TGT GGT CAC G-3'
recA[a]	80F: 5'-GAT CGT ATG GCT CAA GAA G-3'	720R: 5'-GCC ATT GTA GCT GTA CCA AG-3'
16S rDNA[b]	130F: 5'-GTC TAC CAA TGG GTC GTA TC-3'	1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3'

Note: **a** Sawabe et al. 2013; **b** Lane 1991.

PCR reaction conditions

Primer concentrations, magnesium concentrations, dNTP concentrations and amplification conditions are listed in Table 30. It should be noted that each primer set requires different concentrations of reactants and amplification conditions.

Table 30 MLSA primer reaction conditions

Name	Reactants	Amplification conditions
gapA	• Primers 0.3 µM • 1× buffer • 0.8 Mm dNTPs • 1.75 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.

Name	Reactants	Amplification conditions
ftsZ	• Primers 0.3 µM • 1× buffer • 0.8 Mm dNTPs • 1.75 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.
topA	• Primers 0.3 µM • 1× buffer • 0.8 Mm dNTPs • 1.68 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.
mreB	• Primers 0.3 µM • 1× buffer • 0.8 Mm dNTPs • 1.68 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.
gyrB	• Primers 0.4 µM • 1× buffer • 0.8 Mm dNTPs • 1.5 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 36 cycles – 94°C for 20 seconds – 52°for 20 seconds – 72°C for 55 seconds.
rpoA	• Primers 0.3 µM • 1× buffer • 0.8 Mm dNTPs • 1.75 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.
pyrH	Type 1 • Primers 1 µM • 1× buffer • 0.8 Mm dNTPs • 1.63 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.

Name	Reactants	Amplification conditions
recA	• Primers 0.3 µM • 1× buffer • 0.8 Mm dNTPs • 1.75 µM $MgCl_2$ • 1 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 50°for 20 seconds – 72°C for 55 seconds.
16S rDNA	• Primers 0.25 µM • 1× buffer • 0.8 Mm dNTPs • 2 µM $MgCl_2$ • 0.5 U high fidelity Taq.	• 1 cycle – 94°C for 2 minutes • 35 cycles – 94°C for 20 seconds – 60°for 20 seconds – 72°C for 55 seconds.

Approach to data analysis

Data analysis for MLSA is beyond the scope of this ANZSDP. In general terms, as an initial step, the 16S rRNA gene should be sequenced and a phylogenetic tree created to determine the approximate location of the unknown within the family of *Vibrionaceae*. Having established the neighbourhood for the unknown, MLSA should then proceed with reference to its nearest neighbours. Approaches used for the *Vibrionaceae* are described by Sawabe et al. 2013, Balboa and Romalde 2013 or Ashrafi et al. 2015 for *Flavobacterium columnare*.

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Appendix A: Maintenance media

Table 31 Johnson's marine agar

Ingredient	Amount
Peptone (Oxoid LP0037)	5.0 g
Yeast extract	1.0 g
Ferrous (II) sulphate ($FeSO_4 \cdot 7H_2O$)	0.2 g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3 g
Agar	12.0 g
Aged seawater	900 mL
Distilled water	100 mL
pH	7.5 to 7.6

Note: autoclave at 121°C for 15 minutes (Johnson 1968).

Table 32 Sheep blood agar + 2% sodium chloride (NaCl)

Ingredient	Amount
Blood agar base no.2 (Oxoid CM0271)	40.0 g
Sodium chloride (NaCl)	15.0 g
Distilled water	1000 mL
pH	7.4±0.2

Note: autoclave at 121°C for 15 minutes and cool to 50°C; aseptically add 70 mL of sterile defibrinated sheep's blood, mix gently and pour as plates. Horse blood (5%) may be used instead of sheep's blood.

Table 33 *Vibrio* recovery media

Ingredient	Amount
Peptone (Oxoid LP0037)	5.0 g
Yeast extract	1.0 g
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.2 g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3 g
Sodium pyruvate	1.0 g
Bacteriological charcoal	2.0 g
Aged seawater	900 mL

Ingredient	Amount
Distilled water	100 mL
pH	7.5 to 7.6

Note: autoclave at 121°C for 15 minutes (Carson et al. 2006).

Table 34 ZoBell's marine agar 2216E

Ingredient	Amount
Peptone (Oxoid LP0037)	5.0 g
Yeast extract (Oxoid LP0021)	1.0 g
Ferric (III) phosphate ($FePO_4 \cdot 4H_2O$)	0.1 g
Aged seawater	900 mL
Distilled water	100 mL
Agar	12.0 g
pH	7.5 to 7.6

Note: autoclave at 121°C for 15 minutes (Oppenheimer and ZoBell 1952).

Table 35 Cryopreservative (freezing)

Ingredient	Amount
Proteose peptone no.3 (Difco)	1.0 g
Glycerol	8 mL
Distilled water	92 mL

Note: dispense as 5 mL volumes and autoclave at 121°C for 15 minutes (Ward and Watt 1971).

Table 36 Cryopreservative (freeze drying)

Ingredient	Amount
Nutrient broth no.2 (Oxoid CM067B)	16 mL
meso-inositol	2.4 g

Note: filter sterilise and add aseptically to 32 mL of sterile inactivated horse serum. Mix and divide into 3 mL volumes. Store at –20°C. (Furniss et al. 1978).

Appendix B: Culture media for identification

Note, media formulations are based on the cited source references but have been modified in some instances for use with the *Vibrionaceae*. In most cases the modification relates to the addition of sodium chloride (NaCl) and supplementation with yeast extract.

The tests as formulated should not be substituted with variants. The media as described are standardised for the identification of *Vibrionaceae* using the identification matrix VibEx7.

Table 37 Arginine dihydrolase test medium

Ingredient	**Amount**
Peptone (Oxoid LP0037)	0.1 g
Yeast extract (Oxoid LP0021)	0.1 g
Sodium chloride (NaCl)	1.5 g
K_2HPO_4	0.03 g
Phenol red (1% aq)	0.1 mL
L-arginine hydrochloride	1.0 g
Agar	0.3 g
Distilled water	100 mL
pH	6.8

Note: dispense as 4 mL volumes in 12 × 90 mm culture tubes. Autoclave at 121°C for 15 minutes (Thornley 1960, West and Cowell, 1984).

Table 38 Fermentation test medium

Ingredient	**Amount**
Peptone (Oxoid LP0037)	10.0 g
Yeast extract (Oxoid LP0021)	1.0 g
Lab Lemco (Oxoid LP0029)	3.0 g
Sodium chloride (NaCl)	15.0 g
Bromocresol purple 0.5%[a]	8 mL
Distilled water	900 mL
pH	7.2

Note: **a** 0.5 g bromocresol purple in 100 mL 50:50 v/v ethanol/distilled water. Dissolve the ingredients, check and adjust pH and dispense as 90 mL volumes in screw cap bottles. Sterilise by autoclaving at 121°C for 15 minutes. Prepare filter sterilised stocks of the following sugars as 10% stocks in distilled water: arbutin, mannitol, salicin, sucrose and β-gentiobiose. To 90 mL of sterile base add 10 mL of 10% sugar stock to give a

final concentration of 1%. Dispense medium as 3 mL volumes in 12 × 90 mm diameter sterile tubes (Atlas and Parks, 1993).

Table 39 Tolerance to sodium chloride (NaCl) test medium

Ingredient	Amount
7% NaCl	
Tryptone (Oxoid L0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
Sodium chloride (NaCl)	7.0 g
Distilled water	100 mL
pH	7.2
10% NaCl	
Tryptone (Oxoid L0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
Sodium chloride (NaCl)	10.0 g
Distilled water	100 mL
pH	7.2

Note: autoclave at 121°C for 15 minutes as 100 mL volumes in sealed bottles to prevent evaporation. Dispense as 3 mL volumes in 12 × 90 mm sterile tubes (West and Cowell 1984).

Table 40 Amylase test medium

Ingredient	Amount
Nutrient broth no. 2 (Oxoid CM0067)	2.5 g
Sodium chloride (NaCl)	1.0 g
Soluble starch	0.1 g
Agar	1.5 g
Distilled water	100 mL
pH	7.5

Note: dissolve all the ingredients except for the agar. Warm to assist solution of the starch if required. Add the agar. Autoclave at 121°C for 10 minutes. Pour as plates (Bascombe et al. 1973).

Table 41 Acetoin (Voges-Proskauer) test medium

Ingredient	Amount
Tryptone (Oxoid LP0042)	0.7 g
Soya peptone (Oxoid LP0044)	0.5 g
Yeast extract (Oxoid LP0021)	0.1 g
Glucose	1.0 g
Sodium chloride (NaCl)	1.5 g
Agar	0.3 g
Distilled water	100 mL
pH	7.0

Note: dissolve all the ingredients including the agar. Dispense as 3 mL volumes in 12 × 90 mm tubes. Autoclave at 115°C for 10 minutes. Cool as butts (Furniss et al. 1978).

Table 42 Acetoin test, Coblentz reagents

Ingredient	Amount
Reagent 1	
α-naphthol	0.5 g
Ethanol	10 mL
Reagent 2	
Creatine	0.03 g
KOH	4.0 g
Distilled water	10 mL

Note: to store refrigerate reagents 1 and 2. Addtionally, store reagent 1 in a dark bottle (MacFadden, 1980).

Table 43 Gelatin test medium

Ingredient	Amount
Nutrient broth no. 2 (Oxoid CM0067)	2.5 g
Sodium chloride (NaCl)	1.0 g
Gelatin	0.5 g
Agar	1.5 g
Distilled water	100 mL

Ingredient	Amount
pH	7.5

Note: dissolve all the ingredients, except for the agar. Warm to assist solution of the gelatin. Add the agar. Autoclave at 115°C for 20 minutes. Pour as plates (West and Cowell 1984).

Table 44 Gelatin test, saturated ammonium sulphate

Ingredient	Amount
Ammonium sulphate	10 g
Distilled water	10 mL

Note: store at room temperature (Pitt and Dey 1970, Lam and Mutharia 1994).

Table 45 Indole test medium

Ingredient	Amount
Tryptone (Oxoid LP0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
Sodium chloride (NaCl)	1.5 g
L-tryptophan	0.04 g
Distilled water	100 mL
pH	7.5

Note: dissolve ingredients. Dispense as 3 mL volumes in 12 × 90 mm tubes. Autoclave at 121°C for 15 minutes (West and Cowell 1984, Hansen and Sørheim, 1991).

Table 46 Indole test, Kovacs' reagent

Ingredient	Amount
p-dimethylaminobenzaldehyde	2.0 g
pentan-1-ol (*n* amyl alcohol)	30 mL
Concentrated HCl	10 mL

Note: dissolve the aldehyde in the alcohol by gently warming at 50 to 55°C. Cool and slowly add the acid. Protect from light and store at 4°C. Note that iso amyl alcohol is not the same as *n* amyl alcohol. The iso- form of pentanol cannot be used for Kovács' reagent (Barrow and Feltham, 1993).

Table 47 Chromogen test nutrient base

Ingredient	Amount
Tryptone (Oxoid LP0042)	4.0 g
Yeast extract (Oxoid LP0021)	0.4 g
Sodium chloride (NaCl)	6.0 g
Distilled water	300 mL
pH	7.5

Note: dissolve ingredients and dispense as four volumes of 75 mL. Autoclave at 121°C for 15 minutes (Carson et al. 2006, Lowe 1962).

Table 48 Chromogen test phosphate buffer 0.1 M pH 7.5

Ingredient	Amount
Prepare a ×10 stock of 0.1 M buffer	
$NaH_2PO_4 \cdot 2H_2O$	0.245 g
$Na_2HPO_4 \cdot 12H_2O$	3.022 g
Distilled water, made up to	100 mL
pH	7.5
Prepare a stock of 0.01 M buffer, pH 7.5	
0.1 M phosphate buffer	10 mL
Distilled water	90 mL

Note: (Carson et al. 2006, Lowe 1962).

Table 49 Chromogen test phosphate buffer 0.1 M pH 8.0

Ingredient	Amount
Prepare a ×10 stock of 0.1 M buffer	
$NaH_2PO_4 \cdot 2H_2O$	0.083 g
$Na_2HPO_4 \cdot 12H_2O$	3.395 g
Distilled water, made up to	100 mL
pH	8.0
Prepare a stock of 0.01 M buffer, pH 8.0	
0.1 M phosphate buffer	10 mL

Ingredient	Amount
Distilled water	90 mL

Note: (Carson et al. 2006, Lowe 1962).

Table 50 Chromogen test IXP medium

Ingredient	Amount
3-indoxyl phosphate	0.08 g
0.01 M phosphate buffer, pH 8.0	25 mL

Note: filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12 × 90 mm sterile tubes. Protect media from the light (Carson et al. 2006, Lowe 1962).

Table 51 Chromogen test PNPG medium

Ingredient	Amount
4-nitrophenyl α-D-galactopyranoside	0.06 g
0.01 M phosphate buffer, pH 7.5	25 mL

Note: filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12 × 90 mm sterile tubes. Protect media from the light (Carson et al. 2006, Lowe 1962).

Table 52 Chromogen test LNG medium

Ingredient	Amount
L-Glutamic acid 5-(4-nitroanilide)	0.06 g
0.01 M phosphate buffer, pH 7.5	25 mL

Note: filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12 × 90 mm sterile tubes. Protect media from the light (Carson et al. 2006, Lowe 1962).

Table 53 Chromogen test NPS medium

Ingredient	Amount
4-nitrophenyl sulphate	0.04 g
0.01 M phosphate buffer, pH 7.5	25 mL

Note: filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12 × 90 mm sterile tubes. Protect media from the light (Carson et al. 2006, Lowe 1962).

Table 54 Aesculin hydrolysis test medium

Ingredient	Amount
Tryptone (Oxoid LP0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
Sodium chloride (NaCl)	1.5 g
Aesculin	0.1 g
Ferric citrate	0.05 g
Distilled water	100 mL
pH	7.5

Note: dissolve ingredients and dispense as 3 mL volumes in 12 x 90 mm tubes. Autoclave at 115°C for 10 minutes. Protect medium from light (Cowan 1974).

Table 55 Carbon source utilisation test, inorganic nitrogenous base

Ingredient	Amount
Buffer base	
TRIS (basic)	6.1 g
Distilled water	500 mL
pH	7.5
Salts solution	
NH_4Cl	1.0 g
$K_2HPO_4 \cdot 3H_2O$	0.075 g
$FeSO_4 \cdot 7H_2O$	0.028 g
Sodium chloride (NaCl)	11.7 g
$MgSO_4 \cdot 7H_2O$	12.3 g
Potassium chloride (KCl)	0.75 g
Yeast extract (Oxoid LP0021)	0.015 g
$CaCl_2 \cdot H_2O$	1.45 g
Distilled water	400 mL

Note: for the buffer base, adjust the pH with concentrated hydrogen chloride (HCl). For the salts solution, weight out all the salts and combine except for the calcium chloride. Add the distilled water; once the salts are fully dissolved add the calcium chloride. Combine the buffer base and salts solution. Divide the medium into 10 × 90 mL volumes. To each volume add 1.2 g of purified agar (Oxoid LP0028) and autoclave at 121°C for

15 minutes; cool to 55°C. To supplement for nutritionally fastidious strains, add 0.015 g/L Casamino Acids (Difco, 0230-15) to the nitrogenous base (Baumann et al. 1984).

Table 56 Carbon source utilisation test, carbon sources

Number	Carbon substrate
1	α-ketoglutarate
2	Acetate
3	D-alanine
4	Citrate
5	L-citrulline
6	D-galactose
7	D-gluconate
8	D-glucosamine
9	D-glucose
10	D-glucuronate
11	Glycerol
12	α-ketoglutarate
13	L-histidine
14	DL-3-hydroxybutyrate
15	trans-4-hydroxy-L-proline
16	DL-lactate
17	D-lactose
18	Propionate
19	Putrescine
20	Succinate
21	Sucrose
22	Water (control)

Note: Prepare 2% w/v or v/v concentrations in distilled water, of the carbon substrates listed; filter to sterilise. To complete the medium, add 10 mL of carbon source to a 90 mL volume of cooled molten inorganic nitrogenous base. Mix well and pour as plates (Baumann et al. 1984).

Table 57 Decarboxylase test medium

Ingredient	Amount
Difco Decarboxylase broth base (Møller)	300 mL
Yeast extract (Oxoid LP0021)	0.3 g
Sodium chloride (NaCl)	4.5 g
pH	6.5

Note: divide the base into three volumes of 100 mL each. To one volume add 1.0 g of L-lysine and to the second volume 1.0 g of L-ornithine; the third volume is a control. Check and adjust the pH to 6.5 if required. Autoclave at 121°C for 10 minutes. Check the pH of the media and, if required, adjust aseptically with 1 N NaOH or HCl. Aseptically dispense the media as 3 mL volumes into 12 × 90 mm sterile tubes (West and Cowell 1984, Møller 1955, Smibert and Krieg 1994).

Table 58 Urease test medium

Ingredient	Amount
Broth base	
Peptone (Oxoid LP0037)	0.1 g
Glucose	0.1 g
Sodium chloride (NaCl)	1.5 g
Na_2HPO_4 (anhydrous)	0.12 g
KH_2PO_4 (anhydrous)	0.08 g
Phenol red (0.01%)	4 mL
Distilled water	95 mL
pH	6.8
Urea stock (40% w/v)	
Urea	8 g
Distilled water	20 mL

Note: for the broth base, dissolve the ingredients and autoclave as a single volume at 115°C for 20 minutes. For the urea stock, dissolve the urea and filter to sterilise. To complete the medium, add 5 mL of sterile urea stock to cool sterile broth base and mix. Dispense aseptically as 2 mL volumes in 12 × 90 mm sterile tubes (Maslen 1952).

Table 59 Starch test medium, gram's iodine

Ingredient	Amount
Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 mL

Note: dissolve the potassium iodide in 20 mL of water and then add the iodine. Once dissolved, make up to 300 mL with water. Store at room temperature in a dark bottle (Hendrickson 1985).

Shelf life of media

Agar plates have a shelf life of 4 weeks and liquid media have a shelf life of 8 weeks when stored at 2°C to 8°C.

Appendix C: VibEx7 probability matrix for the identification of *Vibrionaceae*

Data as % strains positive		***Aeromonas sobria HG7***	***E. calviensis***	***G. hollisae***	***M. marina***	***M. viscosa***	***A. fischeri* biovar I**	***A fischeri* biovar II**	***A. logei***	***A. salmonicida***	***A. wodanis***	***Ph. angustum***	***Ph. damselae* ssp. *damselae* biovar I**	***Ph. damselae* ssp. *damselae* biovar II**	***Ph. damselae* ssp. *piscicida***	***Ph. iliopiscarium***	***Ph. leiognathi***
Test	*No. strains*	7	4	4	1	8	5	10	2	1	1	1	29	7	5	3	4
Arginine dihydrolase		86	99	1	1	57	1	1	1	1	1	1	99	99	99	99	75
Acid:	Arbutin	14	67	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Mannitol	99	50	1	1	1	40	87	99	99	1	99	1	1	1	33	1
	Salicin	14	75	1	1	1	40	40	1	1	1	1	1	1	1	1	1
	Sucrose	57	75	1	1	1	20	1	1	1	99	1	1	1	1	33	50
	Gentiobiose	14	75	1	1	1	60	90	1	1	1	99	1	1	1	1	1
Growth:	7% NaCl	1	25	99	99	1	1	70	1	99	1	99	86	99	1	99	50
	10% NaCl	1	1	25	99	1	1	1	1	1	1	1	1	1	1	1	1
Amylase		99	99	1	1	99	40	55	1	1	99	1	27	14	20	99	1
Voges Proskauer (Acetoin)		86	1	1	1	1	1	1	1	1	1	1	99	99	99	99	99
Gelatinase		71	50	1	1	99	20	1	1	1	1	1	24	33	20	1	25
Indole		86	99	75	1	1	20	1	1	1	99	1	3	1	1	1	1
IXP alkaline phosphatase		86	50	50	1	13	80	99	99	1	1	99	99	85	20	33	25
PNPG α-D-galactosidase		29	99	1	99	1	1	20	1	1	1	1	3	1	20	1	1
LGN γ-glutamyl transpeptidase		71	50	1	99	99	1	50	99	1	1	1	48	14	20	1	75
NPS sulphatase		14	1	1	99	1	60	99	99	1	1	99	1	1	1	1	25
Aesculin hydrolysis		71	99	1	1	20	80	99	99	1	1	50	31	1	1	1	25
Utilisation: α-ketoglutarate		14	25	50	99	1	1	1	99	1	1	99	3	1	20	1	1

Data as % strains positive		***Aeromonas sobria HG7***	***E. calviensis***	***G. hollisae***	***M. marina***	***M. viscosa***	***A. fischeri* biovar I**	***A fischeri* biovar II**	***A. logei***	***A. salmonicida***	***A. wodanis***	***Ph. angustum***	***Ph. damselae* ssp. *damselae* biovar I**	***Ph. damselae* ssp. *damselae* biovar II**	***Ph. damselae* ssp. *piscicida***	***Ph. iliopiscarium***	***Ph. leiognathi***
Test	*No. strains*	7	4	4	1	8	5	10	2	1	1	1	29	7	5	3	4
	Acetate	29	99	99	99	88	80	1	50	99	99	99	93	1	60	99	50
	Alanine	1	99	99	99	25	20	1	1	1	1	1	10	1	99	1	1
	Citrate	43	99	99	1	1	1	60	99	99	99	99	1	1	1	99	1
	Citrulline	1	99	1	1	38	1	1	1	1	1	1	1	1	1	1	1
	Galactose	57	99	99	1	1	40	99	99	99	1	99	93	28	60	99	25
	Gluconate	99	75	75	99	1	1	20	99	99	1	1	3	14	20	99	99
	Glucosamine	99	99	25	1	50	20	99	99	1	1	99	99	42	60	99	99
	Glucuronate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	33	1
	Glycerol	99	99	75	99	99	40	90	99	99	99	99	99	42	60	99	75
	Histidine	29	50	50	1	88	1	1	1	1	1	1	1	1	1	1	1
	DL-3-hydroxybutyrate	1	99	1	1	1	40	1	1	99	1	1	1	1	1	1	25
	Hydroxyproline	1	75	1	1	1	1	1	1	1	1	99	1	1	1	1	1
	DL-lactate	14	99	99	99	99	1	1	1	1	1	1	79	1	1	1	99
	Lactose	1	1	1	1	1	1	1	1	1	1	1	1	1	20	1	1
	Propionate	14	99	99	1	1	1	1	1	1	1	1	1	1	1	1	1
	Putrescine	14	50	1	1	1	1	1	1	99	1	1	1	1	1	99	1
	Succinate	86	75	75	99	99	40	90	99	99	99	99	89	42	40	99	75
	Sucrose	57	75	1	1	1	60	1	1	99	99	1	1	1	1	33	50
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Data as % strains positive		***Aeromonas sobria HG7***	***E. calviensis***	***G. hollisae***	***M. marina***	***M. viscosa***	***A. fischeri* biovar I**	***A fischeri* biovar II**	***A. logei***	***A. salmonicida***	***A. wodanis***	***Ph. angustum***	***Ph. damselae* ssp. *damselae* biovar I**	***Ph. damselae* ssp. *damselae* biovar II**	***Ph. damselae* ssp. *piscicida***	***Ph. iliopiscarium***	***Ph. leiognathi***
Test	*No. strains*	7	4	4	1	8	5	10	2	1	1	1	29	7	5	3	4
Resistance:	0/129 10 µg	99	1	1	99	38	1	1	1	1	1	1	1	14	1	1	1
	0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Ampicillin 10 µg	57	1	1	1	1	60	80	99	99	1	99	65	14	20	1	75
	Novobiocin 5 µg	86	1	25	1	1	1	10	1	1	1	50	27	14	20	33	50
	Carbenicillin 100 µg	17	1	1	1	1	99	80	99	99	1	99	89	42	20	33	75
Lysine decarboxylase		99	25	1	1	38	20	55	99	1	1	99	62	57	1	99	1
Ornithine decarboxylase		29	1	1	1	1	20	33	99	1	1	1	1	1	1	1	1
Urease		14	1	1	1	1	99	99	1	1	1	99	99	99	1	1	25

Data as % strains positive		***Ph. phosphoreum***	***V. aestuarianus***	***V. agarivorans***	***V. alginolyticus***	***V. anguillarum***	***V. campbellii***	***V. chagasii***	***V. cholerae***	***V. cincinnatiensis***	***V. cyclitrophicus***	***V. diazotrophicus***	***V. fluvialis***	***V. furnissii***	***V. gazogenes***	***V. halioticoli***	***V. harveyi* clade**
Test	No. strains	1	3	7	30	60	4	21	12	3	7	4	9	10	3	3	62
Arginine dihydrolase		99	99	1	3	96	1	95	1	1	99	99	99	99	1	1	15
Acid:	Arbutin	1	1	14	3	1	1	1	1	66	1	99	99	1	99	1	29
	Mannitol	1	99	99	99	98	50	99	99	99	85	99	99	99	33	99	94
	Salicin	1	1	57	20	1	75	5	1	66	1	99	99	1	99	1	79
	Sucrose	1	99	1	99	98	1	43	99	66	99	99	99	99	99	67	65

Data as % strains positive		***Ph. phosphoreum***	***V. aestuarianus***	***V. agarivorans***	***V. alginolyticus***	***V. anguillarum***	***V. campbellii***	***V. chagasii***	***V. cholerae***	***V. cincinnatiensis***	***V. cyclitrophicus***	***V. diazotrophicus***	***V. fluvialis***	***V. furnissii***	***V. gazogenes***	***V. halioticoli***	***V. harveyi* clade**
Test	No. strains	1	3	7	30	60	4	21	12	3	7	4	9	10	3	3	62
	Gentiobiose	1	1	99	1	1	50	1	1	33	14	1	11	1	1	1	95
Growth:	7% NaCl	99	67	29	99	93	99	95	58	99	99	99	99	99	99	67	99
	10% NaCl	1	1	1	99	3	1	10	1	66	1	50	56	90	66	33	5
Amylase		1	99	43	99	96	75	99	75	66	99	25	99	70	99	1	99
Voges Proskauer (Acetoin)		99	1	14	99	94	1	5	58	33	1	1	11	1	99	1	2
Gelatinase		1	67	1	99	77	99	99	67	33	99	25	44	30	99	33	97
Indole		1	99	1	99	93	99	95	99	1	85	99	99	99	1	33	97
IXP alkaline phosphatase		99	99	43	99	88	99	99	92	99	85	99	99	99	50	33	99
PNPG α-D-galactosidase		99	99	99	16	53	1	76	75	99	99	99	33	44	99	99	95
LGN γ-glutamyl transpeptidase		1	1	29	96	15	99	86	99	1	71	99	99	99	1	1	94
NPS sulphatase		1	1	14	40	1	1	99	8	1	71	25	11	1	1	1	60
Aesculin hydrolysis		1	33	99	10	63	99	99	22	99	99	99	88	10	99	33	95
Utilisation: α-ketoglutarate		99	67	1	99	36	99	99	99	1	99	1	99	99	1	1	97
	Acetate	1	67	43	96	51	25	99	99	33	99	99	99	90	99	33	89
	Alanine	1	99	14	99	76	50	99	67	1	99	99	99	99	33	1	99
	Citrate	1	99	1	96	95	99	95	92	99	99	99	99	99	66	1	95
	Citrulline	1	1	1	20	16	25	62	1	1	85	1	56	10	1	1	6
Galactose		99	99	71	13	71	1	99	83	66	85	99	99	99	1	99	73
	Gluconate	1	99	14	99	96	1	67	99	66	99	99	99	99	33	1	99
	Glucosamine	99	99	86	99	95	1	99	99	99	99	75	99	99	33	99	84
	Glucuronate	1	1	1	1	1	1	14	25	1	1	25	78	10	99	1	61
	Glycerol	99	99	1	99	95	99	99	99	99	99	1	99	99	99	1	85

Data as % strains positive		***Ph. phosphoreum***	***V. aestuarianus***	***V. agarivorans***	***V. alginolyticus***	***V. anguillarum***	***V. campbellii***	***V. chagasii***	***V. cholerae***	***V. cincinnatiensis***	***V. cyclitrophicus***	***V. diazotrophicus***	***V. fluvialis***	***V. furnissii***	***V. gazogenes***	***V. halioticoli***	***V. harveyi* clade**
Test	No. strains	1	3	7	30	60	4	21	12	3	7	4	9	10	3	3	62
	Histidine	1	1	1	99	93	1	10	67	1	71	99	89	99	1	1	8
	DL-3-hydroxybutyrate	1	1	1	1	1	1	1	1	1	14	1	78	80	1	1	1
	Hydroxyproline	1	1	1	99	1	1	10	1	1	71	1	11	1	1	1	50
	DL-lactate	99	99	29	99	91	75	99	99	66	99	99	99	99	66	99	99
	Lactose	99	1	1	1	1	1	1	1	1	28	50	1	1	1	1	1
	Propionate	1	67	1	99	6	1	95	58	1	99	75	99	99	66	1	94
	Putrescine	1	1	1	99	1	1	5	1	1	1	99	33	90	1	1	2
	Succinate	99	99	29	99	93	99	99	75	99	99	75	99	90	99	1	97
	Sucrose	1	99	1	99	96	1	43	99	66	99	99	89	99	99	67	65
Oxidase		1	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	99	1	1	1	1	1	33	1	1	1	1	1	1	1
Resistance:	0/129 10 µg	1	1	1	70	3	25	19	8	66	28	25	44	60	99	1	82
	0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	10	1	1	1
	Ampicillin 10 µg	1	1	1	99	91	99	99	25	1	1	1	33	80	1	33	97
	Novobiocin 5 µg	1	67	14	90	11	99	19	17	50	71	75	99	99	99	33	94
	Carbenicillin 100 µg	1	33	1	99	90	99	99	33	1	1	1	11	80	1	33	95
Lysine decarboxylase		1	1	1	99	1	50	5	99	1	28	1	1	1	1	1	98
Ornithine decarboxylase		1	1	1	66	1	1	1	99	1	28	1	11	1	1	1	99
Urease		1	1	1	1	1	1	10	1	1	1	1	1	1	1	1	63

Data as % strains positive		***V. harveyi* biovar II**	***V. ichthyoenteri* biovar I**	***V. ichthyoenteri* biovar II**	***V. lentus***	***V. mediterranei***	***V. metschnikovii***	***V. mimicus***	***V. mytili***	***V. natriegens***	***V. navarrensis***	***V. nereis***	***V. nigripulchritudo***	***V. ordalii***	***V. orientalis***	***V. parahaemolyticus***	***V. pectenicida***
Test	*No. strains*	23	6	3	9	19	5	7	5	12	4	6	3	6	6	18	2
Arginine dihydrolase		1	1	1	99	84	99	1	99	1	1	99	1	1	99	6	99
Acid:	Arbutin	1	1	1	1	1	1	1	25	83	75	1	1	1	1	1	1
	Mannitol	83	60	99	75	99	99	86	99	99	99	67	1	1	99	99	1
	Salicin	4	1	1	1	80	1	1	99	92	50	1	99	1	17	18	1
	Sucrose	1	66	99	13	95	99	1	99	99	99	99	1	99	99	11	1
	Gentiobiose	9	1	1	1	21	1	1	99	99	25	1	50	1	1	1	1
Growth:	7% NaCl	99	33	67	38	74	80	86	99	99	99	99	1	1	67	99	1
	10% NaCl	4	16	1	1	5	40	14	99	75	1	67	1	1	1	89	1
Amylase		99	1	1	38	84	99	1	99	92	99	67	67	50	99	99	99
Voges Proskauer (Acetoin)		75	1	1	1	1	99	1	1	1	1	17	1	1	1	1	1
Gelatinase		83	16	1	63	32	60	71	1	42	75	17	99	99	67	99	1
Indole		99	1	1	63	95	60	99	1	27	99	83	67	1	99	99	1
IXP alkaline phosphatase		99	99	99	75	89	40	86	99	75	99	67	67	40	83	99	99
PNPG α-D-galactosidase		99	1	1	50	99	99	43	75	75	1	1	99	1	67	17	1
LGN γ-glutamyl transpeptidase		87	1	1	38	99	1	99	60	67	50	99	67	99	83	99	1
NPS sulphatase		61	16	33	25	79	1	33	1	58	1	1	1	1	1	56	1
Aesculin hydrolysis		99	66	1	71	95	99	1	99	99	75	1	50	1	20	11	1
Utilisation:	α-ketoglutarate	1	1	1	50	37	1	99	20	67	99	99	99	17	1	89	1
	Acetate	99	83	99	88	99	60	99	99	92	75	99	67	1	67	99	1
	Alanine	99	33	1	75	99	60	99	99	99	75	99	99	67	99	99	99
	Citrate	99	16	1	13	99	40	99	99	92	99	99	99	99	99	94	1
	Citrulline	1	1	1	1	11	1	1	20	83	1	83	1	1	1	17	1

Data as % strains positive		***V. harveyi* biovar II**	***V. ichthyoenteri* biovar I**	***V. ichthyoenteri* biovar II**	***V. lentus***	***V. mediterranei***	***V. metschnikovii***	***V. mimicus***	***V. mytili***	***V. natriegens***	***V. navarrensis***	***V. nereis***	***V. nigripulchritudo***	***V. ordalii***	***V. orientalis***	***V. parahaemolyticus***	***V. pectenicida***
Test	*No. strains*	23	6	3	9	19	5	7	5	12	4	6	3	6	6	18	2
	Galactose	74	1	1	25	99	40	86	80	83	1	33	99	1	67	89	1
	Gluconate	99	99	1	25	5	99	99	99	92	99	99	33	1	99	99	1
	Glucosamine	96	99	99	13	99	20	99	99	99	99	99	99	50	83	99	50
	Glucuronate	13	99	1	1	58	1	86	1	33	25	1	33	1	1	17	1
	Glycerol	83	1	1	38	99	99	99	99	92	99	99	99	33	83	99	99
	Histidine	1	1	1	1	89	1	99	99	99	99	99	99	33	17	99	1
	DL-3-hydroxybutyrate	1	1	1	1	16	1	1	1	83	1	99	99	1	50	6	1
	Hydroxyproline	1	1	1	1	1	1	1	1	25	1	1	1	1	83	83	1
	DL-lactate	99	66	99	63	99	40	99	99	99	99	99	99	17	99	99	99
	Lactose	1	1	1	1	99	40	1	20	8	1	1	99	1	1	1	1
	Propionate	78	16	1	13	99	1	50	99	92	99	99	67	1	67	89	1
	Putrescine	1	1	1	13	99	1	1	1	83	1	83	1	1	83	89	1
	Succinate	99	1	99	50	95	60	99	80	99	99	83	99	1	99	89	50
	Sucrose	4	66	99	1	99	99	1	99	99	99	99	1	83	99	11	1
Oxidase		99	99	99	99	99	1	99	80	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	1	5	1	1	1	8	1	1	1	1	1	1	1
Resistance:	0/129 10 µg	91	16	67	99	1	1	1	99	99	25	33	1	1	1	61	1
	0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	6	1
	Ampicillin 10 µg	1	33	1	13	1	40	14	1	8	50	1	1	50	1	94	50
	Novobiocin 5 µg	74	66	1	1	11	40	57	60	99	75	80	1	1	17	94	1
	Carbenicillin 100 µg	1	33	1	25	42	40	43	1	8	75	1	1	17	1	94	99
Lysine decarboxylase		99	1	1	1	22	60	99	1	1	1	1	1	1	1	99	1

Data as % strains positive		*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II	*V. lentus*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*
Test	*No. strains*	23	6	3	9	19	5	7	5	12	4	6	3	6	6	18	2
Ornithine decarboxylase		1	1	1	13	1	1	99	1	1	1	1	1	1	1	99	1
Urease		1	1	1	1	1	1	1	1	1	1	1	1	1	1	17	1

Data as % strains positive		*V. pelagius* biovar I	*V. pelagius* biovar II	*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis sensu lato*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	10	5	4	12	3	11	37	3	1	8	7	7	5	7	7	6
Arginine dihydrolase		1	1	1	99	99	9	99	99	91	99	99	1	1	99	99	17
Acid:	Arbutin	1	1	1	1	1	1	1	1	1	1	1	71	99	99	1	1
	Mannitol	99	99	1	99	33	10	97	99	22	71	99	71	1	99	99	99
	Salicin	1	1	1	1	1	1	8	1	1	25	99	57	99	99	1	33
	Sucrose	99	80	1	1	99	99	8	1	94	25	99	1	1	86	99	83
	Gentiobiose	1	40	1	1	1	1	1	1	1	1	99	28	40	40	1	1
Growth:	7% NaCl	80	80	1	99	67	36	71	33	1	75	57	85	20	99	99	99
	10% NaCl	1	1	1	99	33	1	11	1	1	1	1	1	1	99	99	83
Amylase		20	80	33	99	67	1	84	99	99	13	99	99	99	99	71	83
Voges Proskauer (Acetoin)		1	1	1	99	1	1	1	1	5	1	1	1	1	14	1	67
Gelatinase		20	80	1	99	67	1	89	99	1	13	99	99	80	14	99	99
Indole		1	99	1	75	1	1	95	66	30	63	99	99	1	99	86	83
IXP alkaline phosphatase		99	99	50	92	99	82	92	99	1	99	86	85	99	99	99	99

Data as % strains positive		***V. pelagius* biovar I**	***V. pelagius* biovar II**	***V. penaeicida***	***V. proteolyticus***	***V. rumoiensis***	***V. scophthalmi***	***V. splendidus* biovar I**	***V. splendidus* biovar II**	***V. tapetis sensu lato***	***V. tasmaniensis***	***V. tubiashii***	***V. vulnificus* biovar I**	***V. vulnificus* biovar II**	**Phenon 6**	**Phenon 8**	**Phenon 10**
Test	*No. strains*	10	5	4	12	3	11	37	3	1	8	7	7	5	7	7	6
PNPG α-D-galactosidase		99	99	99	1	67	9	92	1	86	1	99	99	80	14	29	50
LGN γ-glutamyl transpeptidase		80	99	1	75	99	18	50	99	69	13	99	1	1	99	86	67
NPS sulphatase		70	60	1	1	1	1	79	1	1	99	1	1	1	86	86	50
Aesculin hydrolysis		99	99	1	1	33	99	97	99	1	99	99	42	25	99	1	83
Utilisation: α-ketoglutarate		20	20	99	99	67	9	99	99	99	38	1	99	80	99	99	99
	Acetate	90	80	99	99	67	45	76	99	80	99	86	85	99	99	99	99
	Alanine	99	99	99	99	99	1	97	99	83	99	99	99	99	99	99	99
	Citrate	80	99	99	99	99	91	97	99	97	99	99	99	99	99	99	99
	Citrulline	60	99	1	25	1	1	24	1	1	1	99	1	1	71	14	17
	Galactose	99	99	99	1	99	36	92	1	99	1	99	99	99	1	43	67
	Gluconate	99	99	99	99	67	82	84	1	1	99	99	99	99	99	99	99
	Glucosamine	70	99	99	99	99	99	89	66	99	99	99	99	80	99	86	99
	Glucuronate	1	1	25	1	1	64	21	1	97	1	86	85	80	1	1	17
	Glycerol	99	99	99	99	99	1	99	99	99	99	99	85	99	99	99	99
	Histidine	80	1	99	99	33	1	3	1	1	1	99	28	1	86	99	83
	DL-3-hydroxybutyrate	20	1	25	1	1	18	1	1	1	1	99	1	1	99	1	1
	Hydroxyproline	1	1	1	99	1	1	1	1	1	1	86	1	1	1	1	99
	DL-lactate	99	99	99	99	67	99	97	66	99	99	99	99	99	99	99	99
	Lactose	10	20	99	1	33	1	21	1	1	1	99	1	1	1	1	1
	Propionate	60	80	75	99	33	18	39	33	36	13	86	99	80	99	86	99
	Putrescine	99	99	1	99	1	1	5	1	1	1	29	1	1	99	14	17
	Succinate	99	99	99	99	99	91	99	99	99	99	86	99	99	99	86	99
	Sucrose	99	80	1	8	99	99	3	33	88	25	99	1	1	86	99	83

Data as % strains positive		***V. pelagius* biovar I**	***V. pelagius* biovar II**	***V. penaeicida***	***V. proteolyticus***	***V. rumoiensis***	***V. scophthalmi***	***V. splendidus* biovar I**	***V. splendidus* biovar II**	***V. tapetis sensu lato***	***V. tasmaniensis***	***V. tubiashii***	***V. vulnificus* biovar I**	***V. vulnificus* biovar II**	**Phenon 6**	**Phenon 8**	**Phenon 10**
Test	*No. strains*	10	5	4	12	3	11	37	3	1	8	7	7	5	7	7	6
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10µg	60	1	25	67	1	9	29	99	1	63	14	1	1	1	57	99
	0/129 150µg	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1
	Ampicillin 10µg	10	1	1	58	1	1	3	99	1	1	1	1	1	1	57	99
	Novobiocin 5µg	70	80	1	99	1	27	3	33	1	1	57	57	20	99	57	99
	Carbenicillin 100µg	20	1	1	1	33	1	18	99	1	1	1	1	1	1	71	99
Lysine decarboxylase		1	1	1	8	33	1	1	1	1	1	1	99	60	29	1	83
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	1	99	1	29	1	99
Urease		1	1	1	1	33	1	1	1	1	1	1	1	1	14	1	1

Data as % strains positive		**Phenon 52**	**Phenon 15**	**Phenon 19**	**Phenon 20**	**Phenon 21**	**Phenon 24**	**Phenon 25**	**Phenon 26**	**Phenon 27**	**Phenon 29**	**Phenon 36**	**Phenon 41**	**Phenon 42**	**Phenon 43**	**Phenon 45**	**Phenon 46**
Test	No. strains	3	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6
Arginine dihydrolase		33	99	99	83	88	99	99	99	99	99	50	99	99	99	99	99
Acid:	Arbutin	1	99	1	83	99	1	21	1	1	1	1	1	1	9	1	1
	Mannitol	1	99	99	67	99	99	77	99	99	88	50	1	50	91	25	99
	Salicin	1	1	1	99	99	1	57	1	1	99	1	1	1	27	1	1
	Sucrose	1	99	99	99	99	99	99	99	99	12	1	99	99	82	25	1
	Gentiobiose	1	86	1	83	99	25	57	1	1	1	1	1	1	9	1	1
Growth:	7% NaCl	99	63	25	83	99	99	43	99	88	88	50	83	75	91	25	99
	10% NaCl	67	1	1	1	1	1	1	31	1	1	17	1	1	55	1	1

Data as % strains positive		**Phenon 52**	**Phenon 15**	**Phenon 19**	**Phenon 20**	**Phenon 21**	**Phenon 24**	**Phenon 25**	**Phenon 26**	**Phenon 27**	**Phenon 29**	**Phenon 36**	**Phenon 41**	**Phenon 42**	**Phenon 43**	**Phenon 45**	**Phenon 46**
Test	No. strains	3	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6
Amylase		67	63	99	99	99	99	99	93	94	99	67	99	99	73	50	99
Voges Proskauer (Acetoin)		67	25	1	1	1	1	1	1	1	1	1	17	99	9	1	1
Gelatinase		99	13	1	99	99	75	99	99	99	94	1	67	50	55	25	99
Indole		1	99	99	99	88	1	93	99	99	99	50	33	1	91	25	99
IXP alkaline phosphatase		67	99	25	83	99	99	99	99	99	99	1	83	99	99	99	99
PNPG α-D-galactosidase		33	99	99	17	99	99	99	99	88	99	99	99	1	1	1	1
LGN γ-glutamyl transpeptidase		1	50	99	50	99	1	14	99	82	94	67	99	99	55	25	17
NPS sulphatase		1	88	1	17	88	99	93	99	99	99	1	1	1	55	50	50
Aesculin hydrolysis		99	99	99	67	99	1	99	99	99	99	40	50	1	99	1	99
Utilisation: α-ketoglutarate		1	38	1	99	99	25	99	99	69	99	99	1	1	9	25	99
	Acetate	67	38	75	99	88	99	57	99	88	99	99	99	50	73	99	99
	Alanine	33	13	99	83	99	50	99	99	94	99	83	99	99	99	99	1
	Citrate	33	88	99	83	99	50	99	99	99	94	99	67	75	91	99	1
	Citrulline	1	1	25	40	88	1	1	99	1	99	50	67	25	55	1	1
	Galactose	1	99	99	83	99	99	99	99	88	99	99	99	1	9	50	99
	Gluconate	33	50	1	83	99	99	79	99	94	99	33	83	25	99	75	99
	Glucosamine	67	99	99	99	99	99	99	99	94	99	99	1	99	99	99	99
	Glucuronate	1	13	1	1	1	99	1	6	53	99	17	1	1	1	1	1
	Glycerol	67	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
	Histidine	99	1	1	33	13	1	1	18	12	1	67	17	1	9	1	1
	DL-3-hydroxybutyrate	33	1	75	67	1	1	1	1	6	6	33	1	1	1	1	1
	Hydroxyproline	33	1	1	99	1	1	1	1	1	1	1	83	1	18	1	1
	DL-lactate	33	99	99	99	99	25	99	99	94	99	99	99	99	99	75	99
	Lactose	1	1	25	1	1	1	1	1	6	6	1	1	1	1	1	1
	Propionate	67	1	99	67	99	1	7	68	88	94	83	99	99	91	75	1

Data as % strains positive		Phenon 52	Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46
Test	No. strains	3	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6
	Putrescine	1	99	1	17	1	1	1	6	1	1	33	1	1	9	1	1
	Succinate	1	99	99	83	99	99	93	99	99	99	99	99	99	91	99	99
	Sucrose	99	99	99	99	99	99	99	99	99	18	17	99	99	82	25	1
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	13	1	1	1	1	1	1	1	6	1	1	1	9	1	1
Resistance:	0/129 10 µg	67	1	1	1	1	1	14	1	12	1	1	1	1	1	1	33
	0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Ampicillin 10 µg	33	1	1	1	1	1	1	1	1	1	1	99	75	9	25	1
	Novobiocin 5 µg	99	1	50	17	1	1	1	18	1	1	1	67	99	18	1	1
	Carbenicillin 100 µg	1	1	1	1	13	1	21	6	1	65	1	99	99	9	25	1
Lysine decarboxylase		1	25	1	1	1	1	1	1	1	1	1	1	1	9	1	1
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	1	1	1	9	1	1
Urease		1	1	1	50	1	1	1	6	1	1	1	1	1	1	1	1

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	No. strains	9	4	4	14	5	6
Arginine dihydrolase		11	99	25	1	80	99
Acid:	Arbutin	1	1	1	1	1	1
	Mannitol	99	99	99	1	99	1
	Salicin	13	1	1	7	20	1
	Sucrose	99	1	1	14	99	1
	Gentiobiose	1	25	1	93	1	1
Growth: 7% NaCl		89	75	25	21	20	1

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	No. strains	9	4	4	14	5	6
	10% NaCl	11	1	1	1	1	1
Amylase		1	99	1	1	80	99
Voges Proskauer (Acetoin)		1	1	1	1	99	1
Gelatinase		11	75	1	21	1	99
Indole		99	75	1	7	99	1
IXP alkaline phosphatase		99	99	99	93	1	33
PNPG α-D-galactosidase		1	99	99	99	80	99
LGN γ-glutamyl transpeptidase		33	1	1	1	1	66
NPS sulphatase		44	99	50	99	20	16
Aesculin hydrolysis		56	50	1	93	1	1
Utilisation: α-ketoglutarate		1	25	1	1	80	33
	Acetate	99	50	50	7	99	99
	Alanine	1	99	1	1	80	1
	Citrate	89	75	99	99	99	50
	Citrulline	1	1	1	1	1	1
	Galactose	1	99	99	99	60	83
	Gluconate	1	75	25	7	99	1
	Glucosamine	99	99	99	99	20	99
	Glucuronate	44	75	1	1	1	1
	Glycerol	44	99	99	99	99	99
	Histidine	1	1	1	1	60	50
	DL-3-hydroxybutyrate	89	1	1	1	60	1
	Hydroxyproline	11	1	1	1	1	1
	DL-lactate	99	75	1	1	60	99
	Lactose	1	75	99	99	20	1

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	No. strains	9	4	4	14	5	6
	Propionate	99	1	1	1	40	1
	Putrescine	1	1	1	1	99	1
	Succinate	78	99	99	93	99	99
	Sucrose	99	1	1	7	80	1
Oxidase		99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1
Resistance:	0/129 10 µg	89	1	1	1	40	1
	0/129 150 µg	1	1	1	1	1	1
	Ampicillin 10 µg	11	75	50	14	1	1
	Novobiocin 5 µg	89	1	1	1	50	1
	Carbenicillin 100 µg	11	99	99	71	1	1
Lysine decarboxylase		1	99	99	93	1	1
Ornithine decarboxylase		1	1	1	1	1	1
Urease		1	75	99	99	80	1

Appendix D: Probability matrix for the identification of members of the Harveyi clade

Data as % strains positive		***V. alginolyticus***	***V. campbellii***	***V. harveyi***	***V. harveyi* II**	***V. jasicida***	***V. mytili***	***V. natriegens***	***V. owensii***	***V. parahaemolyticus***	***V. rotiferianus***
Test	No. strains	30	4	30	23	6	5	12	1	18	1
Arginine dihydrolase		3	1	17	1	1	99	1	1	6	1
Acid:	Arbutin	3	1	3	1	99	25	83	1	1	99
	Mannitol	99	50	99	83	99	99	99	99	99	99
	Salicin	20	75	80	4	99	99	92	1	18	99
	Sucrose	99	1	99	1	1	99	99	99	11	99
	Gentiobiose	1	50	93	9	99	99	99	99	1	99
Growth:	7% NaCl	99	99	99	99	99	99	99	99	99	99
	10% NaCl	99	1	3	4	17	99	75	1	89	1
Amylase		99	75	99	99	99	99	92	99	99	99
Voges Proskauer (Acetoin)		99	1	1	75	1	1	1	1	1	1
Gelatinase		99	99	99	83	99	1	42	99	99	99
Indole		99	99	93	99	99	1	27	99	99	99
IXP alkaline phosphatase		99	99	99	99	99	99	75	99	99	99
PNPG α-D-galactosidase		16	1	99	99	99	75	75	99	17	99
LGN γ-glutamyl transpeptidase		96	99	90	87	99	60	67	99	99	99
NPS sulphatase		40	1	43	61	99	1	58	99	56	99
Aesculin hydrolysis		10	99	97	99	99	99	99	99	11	99
Utilisation:	α-ketoglutarate	99	99	99	1	99	20	67	99	89	99
	Acetate	96	25	93	99	66	99	92	99	99	1
	Alanine	99	50	99	99	99	99	99	99	99	99

Data as % strains positive		*V. alginolyticus*	*V. campbellii*	*V. harveyi*	*V. harveyi* II	*V. jasicida*	*V. mytili*	*V. natriegens*	*V. owensii*	*V. parahaemolyticus*	*V. rotiferianus*
Test	No. strains	30	4	30	23	6	5	12	1	18	1
	Citrate	96	99	97	99	99	99	92	99	94	99
	Citrulline	20	25	10	1	1	20	83	1	17	1
	Galactose	13	1	83	74	99	80	83	99	89	1
	Gluconate	99	1	99	99	99	99	92	99	99	99
	Glucosamine	99	1	90	96	99	99	99	99	99	1
	Glucuronate	1	1	73	13	33	1	33	99	17	1
	Glycerol	99	99	93	83	99	99	92	99	99	99
	Histidine	99	1	10	1	1	99	99	1	99	1
	DL-3-hydroxybutyrate	1	1	1	1	1	1	83	1	6	1
	Hydroxyproline	99	1	77	1	1	1	25	1	83	1
	DL-lactate	99	75	99	99	99	99	99	99	99	99
	Lactose	1	1	1	1	1	20	8	1	1	1
	Propionate	99	1	99	78	99	99	92	99	89	99
	Putrescine	99	1	3	1	1	1	83	1	89	1
	Succinate	99	99	99	99	99	80	99	99	89	99
	Sucrose	99	1	99	4	1	99	99	99	11	99
Oxidase		99	99	99	99	99	80	99	99	99	99
Agarolysis		1	1	1	1	1	1	8	1	1	1
Resistance:	0/129 10 µg	70	25	90	91	50	99	99	1	61	99
	0/129 150 µg	1	1	1	1	1	1	1	1	6	1
	Ampicillin 10 µg	99	99	97	1	99	1	8	99	94	99
	Novobiocin 5 µg	90	99	99	74	99	60	99	99	94	1
	Carbenicillin 100 µg	99	99	97	1	99	1	8	99	94	99

Data as % strains positive		***V. alginolyticus***	***V. campbellii***	***V. harveyi***	***V. harveyi* II**	***V. jasicida***	***V. mytili***	***V. natriegens***	***V. owensii***	***V. parahaemolyticus***	***V. rotiferianus***
Test	No. strains	30	4	30	23	6	5	12	1	18	1
Lysine decarboxylase		99	50	99	99	99	1	1	99	99	99
Ornithine decarboxylase		66	1	99	1	99	1	1	99	99	99
Urease		1	1	67	1	99	1	1	1	17	99