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Identification of Putative *Vibrio* Species Isolated from Processed Marine Fish Using Thiosulphate-Citrate-Bile-Sucrose (TCBS) Agar

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Authors' contributions

This work was carried out in collaboration between all authors. Author MNNNSN designed the study, performed the literature searches, wrote the protocol, and wrote the first draft of the manuscript. Author SNV managed the analyses of the study, managed the literature searches, performed the statistical analysis, and proofread the final draft of the manuscript, and PMC directed the first draft of the manuscript, the citation and compiled the reference list. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To identify putative *Vibrio* isolates obtained from processed hake, pilchards and horse mackerel using Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar.
Place and Duration of Study: Sampling during April – June 2005 in Walvis Bay Namibia. Further analyses performed at the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa between 2005 and 2007.
Methodology: The 247 putative *Vibrio* isolates obtained from Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar were initially grouped according to their Gram, oxidase and oxidation-fermentation reactions. Thereafter PCR was used to screen the isolates for genes specific to human pathogenic *Vibrio* species such as *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. This was followed by sequencing the 16S rRNA

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gene for isolates of interest and drawing Neighbour Joining phylogenetic trees based on the data. All atypical isolates were further characterised by a combination of selected phenotypic tests and the *V. alginolyticus* specific collagenase gene PCR.
Results: Of the 247 isolates obtained from TCBS, four were Gram positive. Many of the Gram negative isolates belonged to the family *Enterobacteriaceae* and others were members of the *Pseudomonadaceae*. Of the 91 isolates identified as belonging to the *Vibrionaceae* and related families, *Vibrio alginolyticus* was the dominant *Vibrio* species. None of the bacteria isolated from the processed fish belonged to the human pathogenic *Vibrio* species.
Conclusion: This study again demonstrated that TCBS agar is not selective for the isolation of *Vibrio* species and that a wide range of bacteria could be isolated on this medium when analysing marine fish. The identity of putative *Vibrio* isolates obtained from this medium should therefore be confirmed employing a number of phenotypic and genetic techniques to accurately identify the atypical isolates.

Keywords: TCBS agar; V. alginolyticus; processed marine fish.

1. INTRODUCTION

The family *Vibrionaceae* represents a number of genera including *Vibrio*, *Photobacterium* and *Salinivibrio*. *Vibrio* species are one of the most important groups of bacteria that cause food-borne diseases as a result of the consumption of contaminated fish. There are at least 13 *Vibrio* species pathogenic to humans (*V. cholerae*, *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. funissii, V. fluvialis, V. damsela, V. mimicus, V.hollisae, V. cincinatiencis, V. harveyi* and *V. metchnikovi*) causing cholera, gastroenteritis, septicaemia and wound infections, sometimes resulting in fatal infections [1]. The reported fatality rate for *V. vulnificus* infections is 40-50% [2].

Many vibrios are natural inhabitants of the marine environment, largely in association with fish and zooplankton [3] and may contaminate marine fish during processing [4]. Temperature abuse often results in the uncontrolled proliferation of these bacteria in seafood and represents one of the most common routes of transmission of these pathogens to humans. It is therefore important that these processed marine fish should routinely be screened for the presence of pathogenic *Vibrio* species. Though Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar is the standard media recommended for the selective isolation of *Vibrio* species and was shown to perform better than Thiosulphite-Chloride-Iodide agar [5] it is associated with a number of limitations as reviewed by Shikongo-Nambabi et al. [6].

According to the current trade agreements between Namibia and other countries, especially the EU member states, the detection of *V. cholerae* or any related pathogens in processed fish would result in the rejection of the whole consignment as a sign of non-compliance with the trade agreement. *Vibrio cholerae* is predominantly a sucrose fermenter and accurate identification of these isolates is therefore very important. We have recently reported that when using TCBS agar, sucrose fermenting *Vibrio* spp. were isolated from processed fish at a hake processing factory in Walvis bay, Namibia. These isolates were obtained from the intermediate stage of the processing line while none were detected on the incoming fish samples [7]. It was also noted that the isolated *Vibrio* species were able to form biofilms that defy the action of oxidising agents commonly used in the industry as sanitizers [8]. During this study a comprehensive polyphasic identification scheme was used to identify the 247

sucrose and non-sucrose fermenting bacteria isolated from hake, pilchards and horse mackerel using TCBS agar. Various phenotypic tests such as the Gram stain, oxidase, and Oxidation-Fermentation (O-F) tests were employed. This was followed by amplification of unique genes and sequencing of the 16S rRNA gene. To resolve the identity of some of the isolates, further phenotypic tests and the *V. alginolyticus* specific collagenase PCR were also performed. It was important to accurately identify these isolates so as to help eliminate any doubt with respect to the presence of pathogenic *Vibrio* species and the safety of the Namibian processed marine fish.

This is the first time that a comprehensive identification scheme was used targeting suspect human pathogenic *Vibrio* species isolated from marine fish in Namibia. It was therefore important to accurately identify these isolates so as to help eliminate any doubt with respect to the presence of pathogenic *Vibrio* species and the safety of the Namibian processed marine fish.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

The 247 bacteria included in this study were isolated from processed hake (184 isolates), pilchard (48 isolates), and horse mackerel (15 isolates) analysed at a large fish processing plant in Walvis Bay, Namibia as previously described [7]. Fish homogenates were plated on TCBS agar after pre-enrichment in either alkaline peptone water (APW) pH 8.4 or in 3% NaCl-GSTB (Glucose Salt Teepol Broth).

Four reference strains were also included in the study to serve as positive controls. *Vibrio cholerae* isolate (C453) was obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Type strains of *Vibrio parahaemolyticus* (LMG 2850), *V. vulnificus* (LMG 13545) and *V. alginolyticus* (LMG 4409) were obtained from the Belgium Culture Collection, Laboratorium Voor Microbiologie (BCCM / LMG), University of Ghent, Belgium. All cultures were stored in nutrient broth containing 20% glycerol at -80ºC until analysed.

2.2 Initial Phenotypic Screening

The Gram stain differentiation was performed using the 10% KOH method as was described by Buck [9]. A *Micrococcus* isolate was used as a negative control. The presence of the enzyme cytochrome oxidase and the ability of isolates to utilize glucose under both aerobic and anaerobic conditions (O-F test) were tested according to Quin et al. [10]. If none of the O-F test tubes turned yellow the isolate was regarded as glucose inactive. *Vibrio cholerae* isolate (C453) was used as a positive control for oxidase and O-F tests.

2.3 Polymerase Chain Reaction (PCR) Detection of Specific Genes

The 247 putative *Vibrio* cultures were screened for specific genes associated with the three major human pathogenic *Vibrio* species (*V. cholerae, V. parahaemolyticus* and *V. vulnificus)*. *Vibrio parahaemolyticus* (LMG 2850), *V. vulnificus* (LMG 13545) and *V. cholerae* (C453) were included as positive controls. Isolates that reacted with the *V. parahaemolyticus* specific PCR were also screened for the species specific virulence genes, thermostable direct haemolysin (*tdh*) and thermostable direct haemolysin related haemolysin (*trh*). All 119

isolates that amplified with any of the pathogen specific PCR tests were subjected to 16S rRNA gene sequencing as described by Coenye et al. [11].

2.3.1 DNA extraction

Chromosomal DNA required for PCR or sequencing was extracted as described by Le Roux et al. [12]. Bacterial cultures (either test or reference strains) were grown overnight on Nutrient Agar supplemented with 3% NaCl or on sea water agar (SWA) [13]; [14]. A loopful of the culture was suspended in 500 µℓ of sterile distilled water (SDW). After vortex mixing cells were boiled for 10 minutes. Cell debris was removed by centrifugation at 1000 rpm for 60 seconds. The supernatant (200 µℓ) was aspirated and stored at -20ºC until used. In some cases where cells were difficult to lyse, DNA was extracted with the Qiagen DNA extraction kit according to the manufacturer's instructions.

2.3.2 Oligonucleotide primers

Oligonucleotide primers used to amplify species specific genes, pathogenicity genes and the primers for the16S rRNA amplification and sequencing are listed in Table 1. All primers were synthesised by Inqaba Biotechnology, Pretoria, RSA.

2.3.3 PCR conditions

The PCR cycle targeting the 305 bp fragment of the *ompW* gene of *V. cholerae,* using primers (VIB1 and VIB3), were as described by Nandi et al. [15].The reaction was carried out in a 20 µℓ reaction mixture containing 2 µℓ 10 × PCR Buffer, 25 µM dNTP's (Thermo Scientific), 2.5 mM $MgCl_2$, 0.125 µM of each primer (VIB1 and VIB3), 0.0025 IU/µℓ Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology) and 5 µℓ of template DNA. The rest of the volume was made up using nuclease free water.

For the *Vibrio parahaemolyticus* pR72H fragment, PCR reactions were carried out in 30 µℓ reaction mixtures consisting of 3 µℓ of 10 × PCR buffer, 20 µM of dNTP's (Thermo Scientific), 1.7 mM $MgCl_2$, 3.3 µM of each primer solutions, (VP32 and VP33), 0.001 IU/µℓ Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), and 5 µℓ template DNA. The rest of the volume was filled with nuclease free water. Amplification was performed using the cycle described by Lee et al. [16] and Robert-Pillot et al. [17].

Detection of *V. parahaemolyticus* virulence genes, *tdh* and *trh* was done in a reaction mixture consisting of 5 µℓ 10 × PCR Buffer, 20 µM of dNTP's (Thermo Scientific), 0.15 mM $MgCl_2$, 0.4 µM of each of the primers, (L-trh and R-trh) for the *trh* gene and (L-tdh and R-tdh) for the *tdh* gene, 0.0006 IU/µℓ Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), 2 µℓ of template DNA and nuclease free water to a final volume of 50 µl. The PCR cycle described by Bej et al. [18] was used.

For the amplification of the *V. vulnificus* cytolysin-haemolysin gene, the PCR was performed in a 50 µℓ reaction mixture consisting of 5 µℓ of 10 × PCR Buffer, 40 µM dNTP's (Thermo Scientific), 2 µM of $MgCl_2$, 1 µM of each of the primers, (Choi1 and Choi2), 0.15 IU/µℓ DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), 2 µℓ of test DNA and nuclease free water to the final volume. The PCR amplification cycle followed was described by Lee and Choi [19].

Table 1 Associated gene targets and their primer sequences

Target and amplicon size primers	Primers sequence 5'-3'	Reference
***ompW* for *V. cholerae* (305 bp)**		
VIB1 (Forward)	CACCAAGAAGGTGACTTTAATTGTG	[15]
VIB3 (Reverse)	GGTTTGTCGAATTAGCTTCACC	[15]
pR72H for *V. parahaemolyticus* (387 or 320 bp)		
VP32 (Forward)	TGCG AATTCGATAGGGTGTTAACC	[16
VP33 (Reverse)	CGAATCCTTGAACATACGCAGC	[17
Cytolysin-haemolysin for *V. vulnificus* (704 bp)		
Choi1 (Forward)	GACTATCGCATCAACAACCG	[19]
Choi2 (Reverse)	AGGCGAGTATTACTGCCGTA	[19]
16S rRNA (1458 bp)		
pA (Forward)	AGAGTTTGATCCTGGCTCAG	[11]
MH2 (Reverse)	TACCTTGTTACGACTTCACCCCA	[11]
16S rRNA - sequencing		
*pD (16F536)	CAGCAGCCGCGGTAATAC	[11
Collagenase for *V. alginolyticus* (737 bp)		
VA-F (Forward)	CGAGTACAGTCACTTGAAAGC	[20]
VA-R (Reverse)	CACAACAGAACTCGCGTTACC	[20]
Thermostable direct haemolysin (*tdh*) for *V. parahaemolyticus* (849 bp)		
L-tdh (Forward)	GTAAAGGTCTCTGACTTTTGGAC	[18]
R-tdh (Reverse)	TGGAATAGAACCTTCATCTTCACC	[18]
TDH-related haemolysin for *V. parahaemolyticus* (500 bp)		
L-trh (Forward)	TTGGCTTCGATATTTTCAGTATCT	[18]
R-trh (Reverse)	CATAACAAACATATGCCCATTTCC	[18]

For the *V. alginolyticus* collagenase gene, PCR amplification of the gene was carried out in a reaction mixture consisting of 5 µℓ 10 × PCR Buffer, 20 µM dNTP's (Thermo Scientific), 1.5 µM $MgCl_2$, 0.2 µM of each of the Primers, (VA-F and VA-R), 0.001 IU/µℓ of Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), 3 µℓ of DNA. The volume was made up to 50 µℓ with nuclease free water. The PCR protocol described by Di Pinto et al. [20] was used.

The 16S rRNA gene was amplified in a total volume of 50 µℓ, consisting of 5 µℓ of 10× PCR buffer, 2 µM of dNTP's (Thermo Scientific), 2 mM of $MgCl_2$, 5 µM of each primer, 0.0005 IU/µℓ of Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology) 2 µℓ of template DNA and nuclease free water to make up the volume. The PCR cycle described by Coenye et al. [11] was followed. When required bands of the desired size (1458 bp) were cut out of the gel and purified using the Qiagen Gel purification kit.

In all PCR reactions the appropriate type strain was included as the positive control. The nuclease free water was used instead of DNA in the negative control. All PCR mixtures were held at 4ºC after the final primer extension step. The DNA amplicons were observed by running the PCR reactions on 1.5% (w/v) agarose gel (Amersham Pharmacia Biotech) in

1×TE (Tris phosphate EDTA) buffer at 80 V, 200 mA and 100 W for 40 – 45 minutes. Bands were observed using an Upland CA 9178 USA transilluminator Model M5. Gel photos were taken using the Vilber Lourmat camera with Vida max screen.

The 16S rRNA gene sequencing PCR was carried out in a 10 µℓ reaction mixture consisting of the following reagents. Nuclease free water (5 µℓ), 1 µℓ of 5× sequencing buffer, 1 µℓ of the sequencing primer *pD (Forward), 2 µℓ of Big dye Chain Terminator mixture and 1 µℓ template DNA to approximately a final concentration of 150 ng. The PCR cycle consisted of initial denaturation at 96ºC for 5 seconds, a final extension at 60ºC for 4 minutes, and 25 cycles, each consisting of denaturation at 96ºC for 10 seconds, primer annealing at 55ºC for 5 seconds and the primer extension at 60ºC for 4 minutes.

2.4 Sequence Alignment and Constructing of Phylogenetic Trees

After all the partial 16S rRNA sequences were trimmed to yield a 591 bp sequence, two trees were constructed using the Neighbour–Joining (NJ) method to group the unknown isolates. One tree contained 80 *Vibrionaceae* related isolates and the second tree consisted of 30 isolates belonging to the *Enterobacteriaceae*. Sequence alignments were carried out using ClustalX software [21] and the sequences were edited and trimmed with Bioedit Sequence alignment Editor v. 5.0.9 [22]. Phylogenetic trees of the 16S rRNA gene sequences were draw with PAUP (Phylogenetic analysis using Parsimony) [23] using the NJ method [24]. Confidence limits were determined based on the calculated Bootstrap support [25]. Thirty-one sequences of known species, mainly type strains, obtained from GenBank (http://www.cnbi.nlm.nih.gov/BLAST/) were included in the phylogenetic trees to assist with the final grouping of the unknown sequences.

2.5 Analytical Profile Index (API) 20E and other Biochemical Tests

Due to unresolved identifications for many of the isolates, based on the 16S rRNA sequencing, further phenotypic data were collected for these isolates. The tests were specifically selected to help differentiate between *V. parahaemolyticus* and *V. alginolyticus* strains [26]; [27]. The API 20E test was done according to the supplier's instructions and proposed species identification was obtained using the API lab software. Special attention was given to the Voges Proskauer (VP) test, ornithine decarboxylase activity and arabinose fermentation. Other tests included the cellobiose fermentation test determined according to Jones and Wise [28] in a sterile 1% cellobiose solution supplemented with 3% NaCl and 0.15% bromo-cresol purple as pH indicator. The ability to growth in nutrient broth containing 10% sodium chloride [26]; [27] was also tested. *V. parahaemolyticus* LMG 2850 and *V. alginolyticus* LMG 4409 were used as controls.

2.6 Phenotypic Comparison

The biochemical profiles of 52 selected isolates that clustered within the *V. parahaemolyticus / V. alginolyticus* group were imported into Bionumerics. The profile consisted of the 20 miniaturized biochemical tests of API 20E, results for cellobiose and sucrose fermentation, the oxidase test as wells as their ability to growth in nutrient broth containing 10% sodium chloride. A dendrogram was constructed using the Jaccard coefficient and unweighted pair group with arithmetic average (UPGMA) to demarcate isolates as either *V. parahaemolyticus* or *V. alginolyticus* [29]. Data for *V. alginolyticus* LMG 4409 and *V. parahaemolyticus* LMG 2850 were also included.

3. RESULTS AND DISCUSSION

Vibrio species are one of the most important groups of bacteria that cause food-borne diseases as a result of the consumption of contaminated fish. From a consumer health perspective it is therefore important to establish whether these bacteria are present when the microbial quality of the processed fish is determined. TCBS is one of the recommended media for the selective isolation of *Vibrio* species [30]. The selectivity of this medium, may, however, vary [31 and it is important that the identity of the putative *Vibrio* isolates be verified.

The identification of *Vibrio* species is commonly done using classical phenotypic methods and in some cases 16S rRNA sequence comparisons are also included [1]; [32]. Classical techniques are, however, slow and sometimes inaccurate as some strains exhibit atypical phenotypic characteristics. Molecular methods targeting and amplifying unique genes with PCR have also been used, sometimes with limited success [1]. During this study a comprehensive polyphasic scheme, combining several of these tests, was used to determine the identity of the isolates recovered from TCBS agar.

Bacteria other than *Vibrio* such as *Enterobacteriaceae*, *Proteus*, *Aeromonas* and *Staphylococci* may also grow on TCBS [33]. Vibrios form characteristic colonies (yellow or green) based on the ability of the isolate to ferment sucrose. Pathogenic *Vibrio* species belong to both groups and variability within a species with regard to sucrose fermentation is common. Sucrose fermentation is therefore not an important trait and could not be used to differentiate *Vibrio* species from *Enterobacteriaceae* or *Pseudomonadaceae*.

3.1 Initial Phenotypic Characterization

When the quality of processed fish was determined in a large processing plant 247 isolates growing on TCBS agar, were selected for further identification. The majority of the isolates (243) formed the characteristic string in the KOH test and was confirmed as Gram negative bacteria [34]. Only four (1.6%) isolates were Gram positive, confirming that TCBS is not 100% selective for Gram negative bacteria as was also reported by Gomez-Gil and Roque [35].

Both sucrose and non-sucrose fermenting pathogenic *Vibrio* species are often transmitted to humans via consumption of fish or through sea water [1]. Strains with atypical sucrose fermentation patterns have also been reported for all three of the human pathogenic *Vibrio* species targeted in this study. A few strains of *V. parahaemolyticus* [33] and *V. vulnificus* [36]; [37]; [38] have been reported to ferment sucrose. On the other hand sucrose negative *V. cholerae* O139 strains have also been documented [39]. For this reason both sucrose and non-sucrose fermenting colonies were included in this study. Out of the 243 Gram negative isolates, 133 were sucrose fermenters while 110 were non-sucrose fermenters.

Based on the oxidase and O-F tests performed on the 243 isolates (Fig. 1) only 79 (32.5%) of the isolates were oxidase positive and could ferment glucose indicating clearly that they belong to the family *Vibrionaceae* (including genera such as *Vibrio*, *Aeromonas*, *Shewanella*). Eighty (32.9%) of the isolates were oxidase negative facultative anaerobic bacteria and were grouped into the family *Enterobacteriaceae.* Eight (3.2%) of the isolates were oxidase positive and could only utilize glucose aerobically, indicating that they possibly belonged to the family *Pseudomonadaceae*. Thirteen (5.3%) of the isolates were oxidase

positive, but gave weak results in the O-F test. Ten (4.1%) were oxidase negative and glucose inactive and were suspected to be members of the *Enterobacteriaceae*. A further 53 (21.8%) isolates were oxidase positive but also did not utilize glucose. The inability of these isolates to ferment glucose suggests the atypical phenotypic characteristics of some of the bacteria isolated from marine waters. It again highlights some of the difficulties encountered during the identification of some environmental isolates when only using phenotypically based schemes.

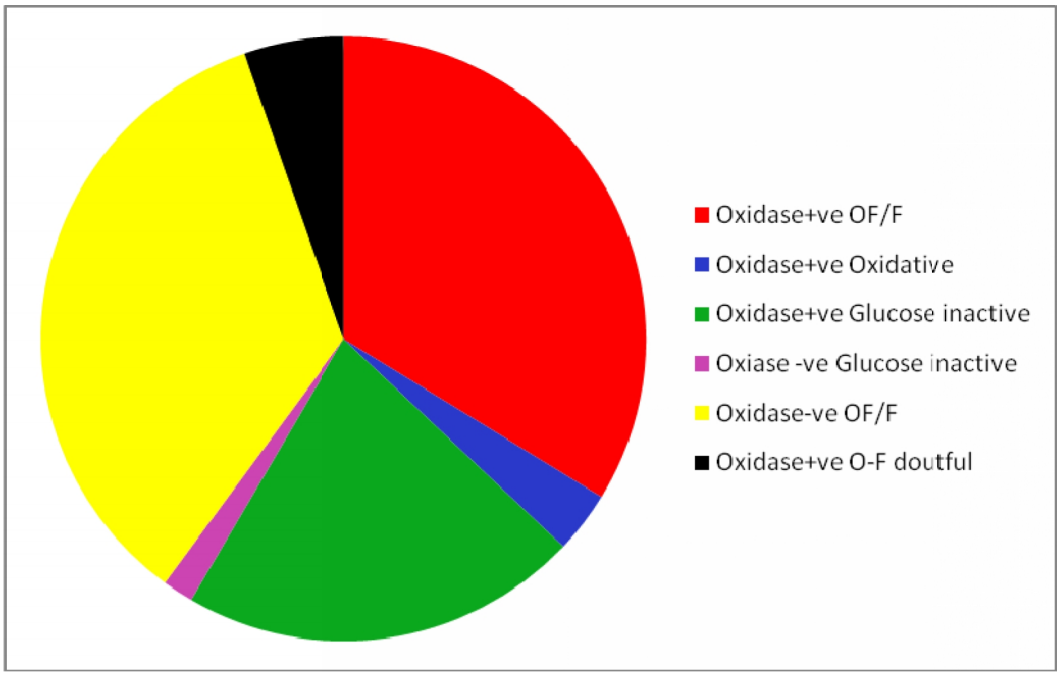

Fig. 1. Grouping of the 243 Gram negative isolates from TCBS on the basis of their cytochrome oxidase reaction and the ability of isolates to utilize glucose under both aerobic and anaerobic conditions (O-F test)

3.2 Detection of Species Specific Genes

Due to the atypically characteristics of the bacteria isolated from TCBS agar (e.g. the inability to ferment glucose) and because of problems experienced in the past by other researchers to accurately identify *Vibrio* species to species level [40]; [41] all the isolates were screened for the presence of genes typically associated with the human pathogenic *Vibrio spp*.. When screened with the selected species specific primers many isolates reacted with the *V. parahaemolyticus* and *V. vulnificus* specific primer pairs and some isolates even reacted with both. Amongst these isolates some showed either non-specific reactions or the formation of atypical sized bands.

Fifty-seven (23.5%) isolates amplified with the VP32/VP33 primer pair targeting the pR72H fragment of *V. parahaemolyticus*. Thirty three of these isolates produced two bands of 387 and 450 bp in size while 24 produced the expected single band of 387 bp.

Eighty-eight (36.2%) of all the isolates amplified with the *V. vulnificus* specific Choi1/Choi2 primer pair targeting the cytolysin / haemolysin gene. Of the 88 Choi1/Choi2 primer pair positive isolates, thirty-seven formed a band of 704 bp which was similar in size to the band formed by the positive control *V. vulnificus* LMG 13545 strains. Twenty-five isolates formed bands smaller than that of the positive control and eleven formed a band larger than the typical 704 bp product. Another fifteen isolates formed bands either equal and greater or equal and smaller to the band produced by the positive control. Eighteen of these isolates also amplified with the *V. parahaemolyticus* specific primers.

Only 7 (12.1%) of the 57 isolates were positive for both the pR72H and the *trh* gene fragments. Good amplification were observed although these bands were all larger than the typical 269 bp product obtained from the *V. parahaemolyticus* type stain. The bands obtained from the isolates were about 300 bp and 350 bp in size. None of the isolates reacted with the R-tdh/L-tdh primer pair.

Positive reactions for the pR72H fragment (Phosphatidyl serine synthetase gene) [16] were detected for a number of isolates later identified as *V. alginolyticus* and *Shewanella* species. The *V. vulnificus* specific cytolysin-haemolysin gene [19] was detected amongst isolates later identified as belonging to various genera including *Shewanella*, *Aeromonas, Pseudomonas*, *Providencia*, *Morganella* and *Citrobacter*. *V. alginolyticus* isolates were the predominant group for which both genes could be amplified. The reaction of *V. alginolyticus* strains to *V. parahaemolyticus* specific genes has confirmed previous findings where this was observed [42]; [17]; [43]. This study has shown that these two genes are either widely distributed amongst different environmental isolates or that the PCR assays are not as specific as has initially been reported. Tests developed to accurately identify clinical strains might therefore not be appropriate for bacteria isolated from the environment.

3.3 16S rRNA Gene Sequence Analysis

The 16S rRNA genes of 119 of the isolates that amplified with either the VP32/VP33 and/or the Choi1/Choi2 primers pairs were sequenced. A Basic local alignment search tool for nucleotides (BLASTn) search was conducted to compare the partial 16S rRNA sequences (591 bp) of these isolates to the sequences in Genbank. Eighty-three (69.7%) of the isolates aligned within the family *Vibrionaceae*, 31(26.1%) with the *Enterobacteriaceae* and five of the isolates could be grouped with *Pseudomonas* species. Of the 83 isolates associated with the *Vibrionaceae,* 66 isolates aligned closely to *Vibrio*, 13 to *Shewanella*, and four to *Aeromonas*. Of the isolates that grouped within the genus *Vibrio*, 22 closely aligned to *V. parahaemolyticus* and 31 to *V. alginolyticus*.

The NJ analysis (Fig. 2) assigned the *Vibrionaceae* sequences to 14 clusters. Fifty-eight isolates grouped in cluster 1 together with the type strains of *V. alginolyticus, V. campbellii, V. natriegens, V. mytili, V. rotiferianus, V. diabolicus, V. parahaemolyticus* and *V. nereis.* Four isolates grouped in cluster 2 and one isolate remained separate (cluster 3). The *V. fluvialis*, *V. harveyi* and *V. proteolyticus* type strains each formed a separate cluster. Cluster 7 consisted of the *V. albensis*, *V. cholerae* and *V. mimicus* types. None of the fish isolates were found in these clusters. One isolate clustered within the *Listonella anguillarum* group (cluster 8). Cluster 9 consisted of eight isolates that belonged to the genus *Shewanella* but that did not group with any of the known *Shewanella* type strains. Three isolates clustered together with *Shewanella haliotis* in Cluster 10. One of the isolates was found within cluster 11 together with *Shewanella algae*. Cluster 13 was a distinct group consisting of *Aeromonas veronii* and three of the isolates, cluster14 consisted of the *Aeromonas hydrophyla* type

strain which grouped with one of the isolates. Most of these groups were supported with bootstrap values of between 55-100%. *Aeromonas punctata* was used as an outgroup. Most of the isolates could not be identified to species level due to the high level of interspecies 16S rRNA nucleotide sequence homology displayed within the genus *Vibrio* [44].

Based on the NJ analysis the *Enterobacteriaceae* isolates could be divided into four groups (Fig. 3). Fifteen isolates clustered together with the type strains of *Providencia alcalifaciens*, *Providencia rustigianii*, *Providencia heimbachae* and *Providencia rettgeri* in Cluster 1. Ten isolates clustered with *Morganella morganii* subspecies *morganii* and *Morganella morganii* subspecies *sibonii* in Cluster 2. Four isolates clustered with *Citrobacter braakii*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Citrobacter werkmanii* in Cluster 3. One isolate grouped with *Enterobacter hormaechei in* Cluster 4. The four clusters were supported by bootstrap values of between 52 and 98. *Serratia marcescens* was used as the outgroup (Fig. 3).

3.4 Biochemical Profiles

Using the API 20E system, further biochemical reactions were determined for the 63 isolates for which it was impossible to distinguish between *V. alginolyticus* or *V. parahaemolyticus* in the NJ tree (Fig. 2). Evaluation of the API 20E data, using the API lab software database, we identified 58 of the strains as *V. alginolyticus,* four as *A. hydrophyla* and one as *Pasteurella pneumotropica*. Identification of *Vibrio* species based on limited phenotypic tests is difficult. This is especially true for the differentiation between *V. alginolyticus* and *Vibrio parahaemolyticus* as these two species share many of the characteristics. The most common test used to differentiate between these species is their ability to ferment sucrose, but final identification is based on the VP, urease, cellobiose, dulcitol, sucrose, ONPG, arabinose test and growth in the presence of 10-12% NaCl [26]; [27]. Twenty-six of the *V. alginolyticus* isolates identified by the API system differed from the typical *V. alginolyticus* biochemical profile in terms of the VP test, cellobiose and amygdalin fermentation and lysine decarboxylase (LDC). One of the isolates also did not ferment sucrose.

To assess the overall phenotypic similarity of the isolates a dendrogram based on their biochemical profiles were constructed using the Jaccard coefficient (Fig. 4). All isolates clustered closer to *V. alginolyticus* LMG 4409 and were clearly distinct from *V. parahaemolyticus* LMG 2850. Isolate 406 differed substantially from the other *V. alginolyticus* strains as it fermented arabinose, produced urease and H_2S. It was also TDA (tryptophan deaminase) positive and the isolate did not produce indole from tryptophan. Overall assessment of the phenotypic data provided the best means to differentiate between *V. parahaemolyticus* and *V. alginolyticus*. This finding is in line with the study of West et al. [45] who used the same set of biochemical characters and were able to clearly differentiate the two species.

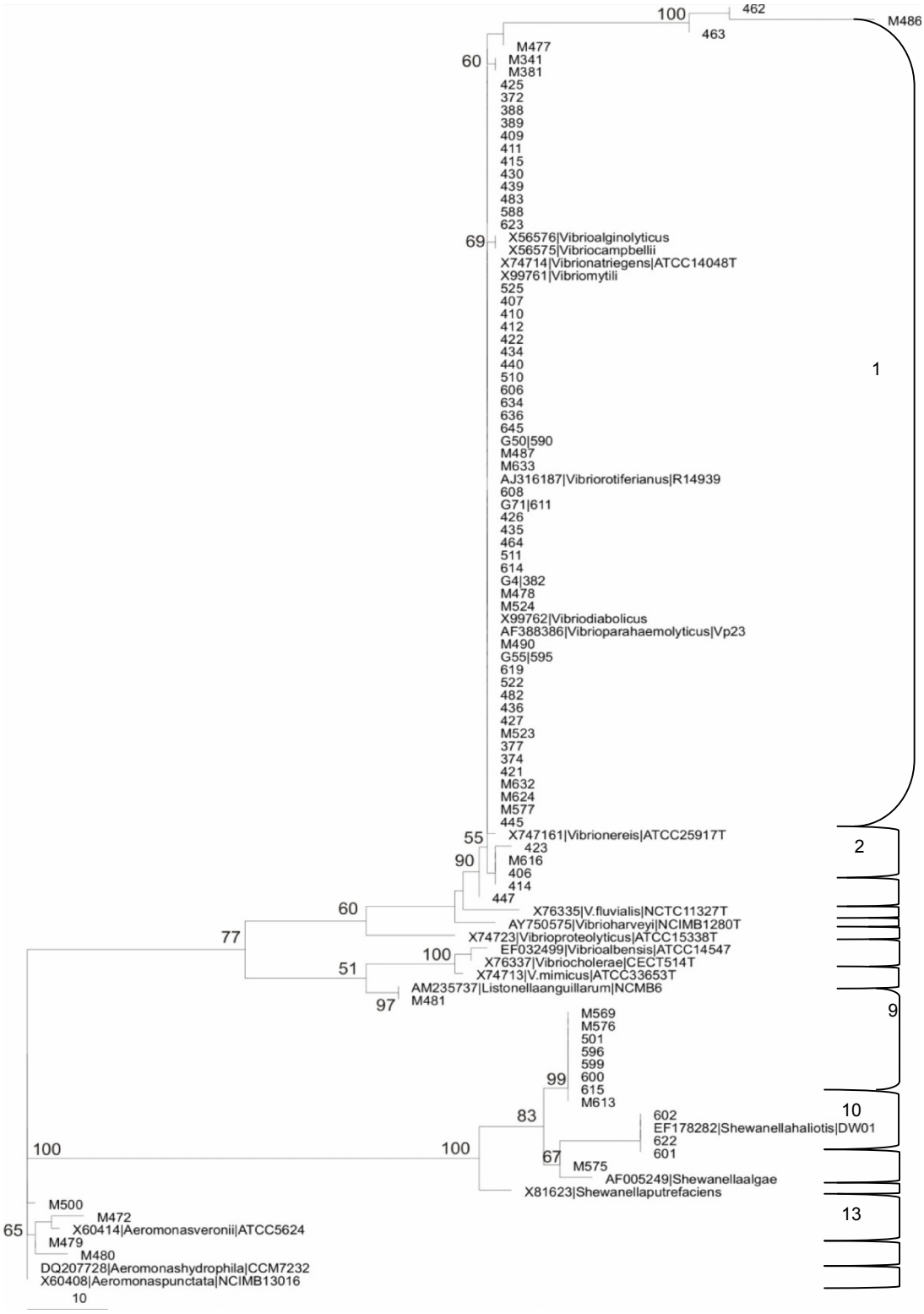

Fig. 2. Neighbour-joining trees of 80 isolates and 21 type strains belonging to the *Vibrionaceae* based their partial 16S rRNA sequences. Bootstrap values were based on a 1000 repeats. *Aeromonas punctata* was used as an outgroup

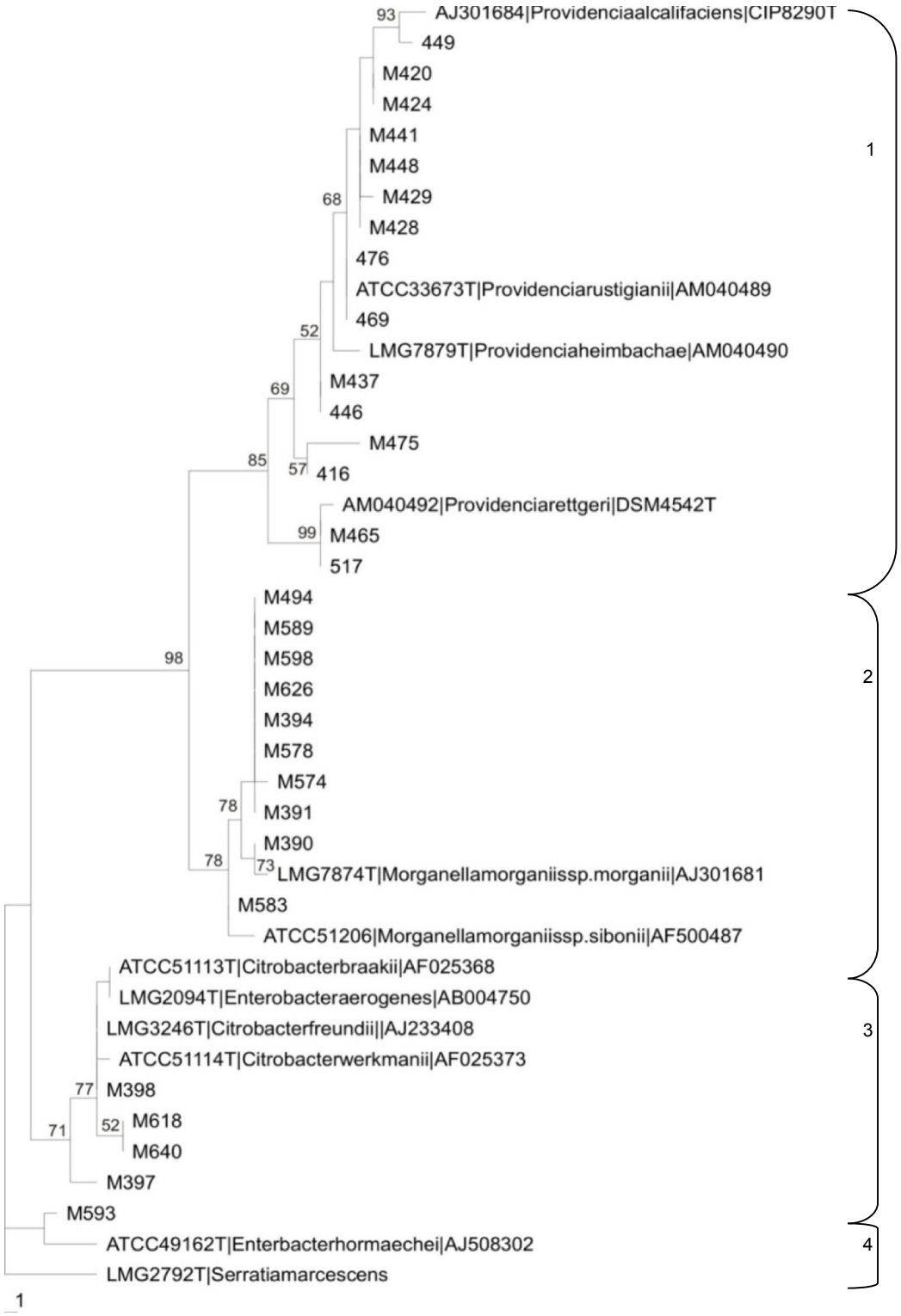

Fig. 3. Neighbour-joining tree of 30 isolates and 12 type strains belonging to the *Enterobacteriacea* based on their partial 16S rRNA sequences. Bootstrap values were based on a 1000 repeats. *Serratia marcescens* was used as an outgroup

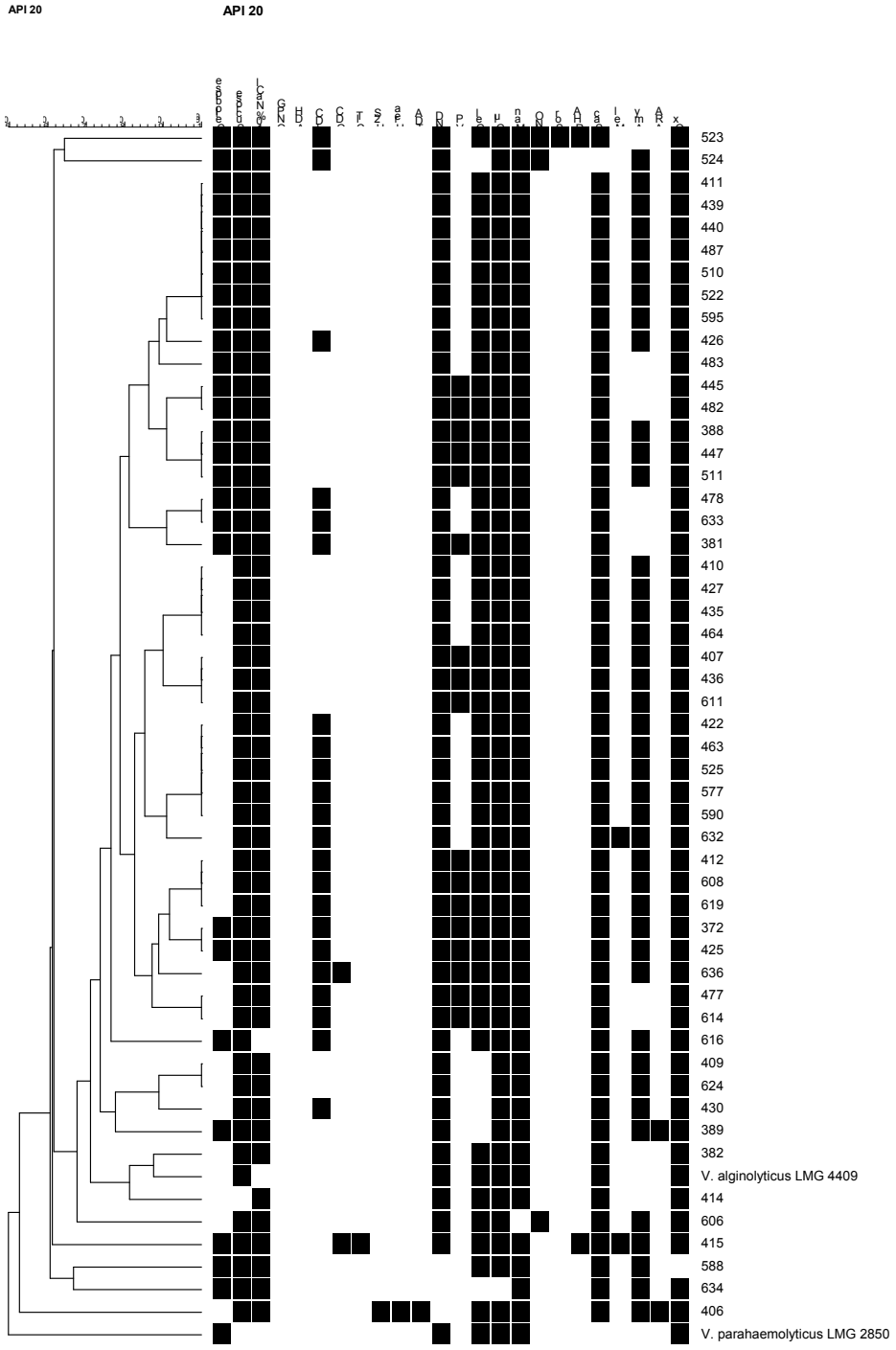

Fig. 4. Dendrogram showing the levels of similarity amongst the biochemical profiles of 52 selected *Vibrio* isolates

3.5 *V. alginolyticus* Specific Collagenase PCR

Twenty two of the isolates showed a high similarity with *V. parahaemolyticus* when their partial 16S rRNA sequences were compared during a BLASTn search on GenBank. These strains were, however, identified as *V. alginolyticus* based on their overall biochemical profiles. To confirm this identification these isolates were screened for the *V. alginolyticus* specific collagenase gene. Nineteen of these isolates produced a 737 bp fragment similar to that of *V. alginolyticus* LMG 4409, and their identity could be confirmed. Only three of the isolates as well as the type strains of *V. parahaemolyticus* LMG 2850*, V. vulnificus* LMG 13545 and an environmental strain of *V. cholerae* C453 did not react with the *V. alginolyticus* collagenase primers. The three isolates could still belong to *V. alginolyticus* as Cai et al. [46] reported that this gene is mainly associated with *V. alginolyticus* strains that are pathogenic to fish.

3.6 Identities of Isolates Growing on TCBS Agar

Of the 247 isolates originally selected from TCBS agar, 80 were potentially members of the *Enterobacteriaceae* based on the original phenotypic data. Based on the 16S rRNA data collected for some of these isolates, 15 isolates belonged to the genus *Providencia*, 10 to the genus *Morganella* and a further four to *Citrobacter*. Eight of the isolates belonged to the *Pseudomonadaceae* and another 43 of the isolates that did not utilize glucose could also belong to this group. Only four of the isolates were identified as Gram positive bacteria.

Less than half of the isolates (91) were identified as belonging to the *Vibrionaceae* and related families, based on the initial phenotypic data. Subsequently, 51 were identified as *V. alginolyticus* based on their overall biochemical profiles and other supporting data. Ten of these isolates had atypical reactions as they did not ferment glucose during the initial the O-F test. Amongst the other isolates initially grouped with the *Vibrionaceae* and related families, a further 12 isolates were shown to belong to the genus *Vibrio*, 12 isolates grouped with *Shewanella*, and 4 could be linked to *Aeromonas* based on their partial 16S rRNA sequence data.

None of isolates initially isolated from the processed using TCBS agar belonged to the human pathogenic *Vibrio* species. Although none of the identified isolates were confirmed to be *V. parahaemolyticus* the high phenotypic and genetic similarity of *V. alginolyticus* isolates to *V. parahaemolyticus* could perhaps indicate that the virulent strains of *V. parahaemolyticus* would be able to thrive in processed marine fish under similar conditions and that the source of these contaminants during processing should be addressed.

4. CONCLUSION

None of the 247 bacteria isolated from hake, pilchard or horse mackerel was identified as *V. cholerae, V. parahaemolyticus* or *V. vulnificus*. The study implies that these samples were safe and did not pose a health hazard to consumers. Based on the tests evaluated during this study, no rapid test to confirm the identity of these pathogens could be identified. The *V. parahaemolyticus* the pR72H fragment and *V. vulnificus* cytolysin haemolysin gene primers used were not specific for the detection of these pathogens. Many of the environmental isolates, including *Providencia*, *Morganella Citrobacter* and *Shewanella isolates* showed cross reactivity with these primers and these assays should not be used for the routine monitoring of seafood samples in their present format.

It has again been demonstrated that when isolates belonging to either *V. alginolyticus* or *V. parahaemolyticus* need to be identified, a polyphasic approach using various phenotypic and genetics tests should be used. Neither the classical methods nor the partial 16S rRNA sequence alone could differentiate between these two species. The 16S rRNA sequences were only able used to differentiate the *V. parahaemolyticus* / *V. alginolyticus* group from the other pathogenic species such as *V. cholerae* and *V. mimicus*. The 16S rRNA results in combination with biochemical profiles based on the API 20E data, facilitated accurate identification of the *Vibrio* isolates. Screening for the collagenase gene of *V. alginolyticus* may also help to avoid ambiguity in the final results.

This study confirmed that care should be taken when using TCBS agar to determine the presence of *Vibrio* species in processed seafood. Many of the putative *Vibrio* isolates obtained during this study did not belong to this group of bacteria and the selectivity of TCBS needs to be improved to minimise growth of *Pseudomonas, Aeromonas*, *Shewanella* and members of the *Enterobacteriaceae*. When using this medium during the routine analysis of processed fish, the identity of selected strains should be determined on a regular basis to ensure accurate reporting of the microbial quality of the seafood.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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