CHAPTER 3

3.1 ABSTRACT

Problems with the safety and shelf life of hake intended for export have been raised in the Namibian fishing industry. This prompted an investigation into the factors that may have an impact on the microbiological quality and safety of processed hake. Samples were collected at three stages along the processing line; the general microbiological quality (mesophilic and psychrotrophic aerobic plate counts), total *Vibrio* species and common fish spoilage bacterial counts were performed. The results constantly showed relatively high counts for the psychrotrophic and spoilage bacteria, indicating that most of these bacteria already formed part of the incoming fish. Hake is headed and gutted on board of fishing vessels and delivered to the factory only after 7–8 days for final processing. It is likely that this practise of heading and gutting the hake may have a negative effect on microbiological quality of the final product. A sharp increase in the mesophilic and sucrose fermenting *Vibrio* species counts were observed after filleting. It has been suggested that this contamination could be due to biofilms present in the distribution system for the treated sea-water used during processing. Although sea-water could be an alternative source of water for marine fish processing plants, the treatment and the quality of the water needs to be carefully managed.

Key Words: hake fish, aerobic plate counts, *Vibrio* species, mesophiles, psychrophiles, spoilage bacteria.

3.2 LIST OF ABBREVIATIONS

AA:	aeromonas agar
ABM-F:	after filleting machine hake fillets washed with fresh water
ABM-S:	after filleting machine hake fillets washed with sea-water
APW:	alkaline peptone water pH 8.4
APHA:	American Public Health Association
BM:	basal medium
CFC:	cetrimide fusidin cephaloridin agar
EU:	European Union
FP:	hake fish finished products
H and G:	headed and gutted hake fish
ICMSF:	international commission on microbiological specifications for foods of the international association of microbiological societies
LFHF:	laminated and folded hake fillets
PCA:	plate count agar
PW:	peptone water
SWA:	sea water agar
TVC:	total viable counts
TCBS:	thiosulphate citrate bile salts sucrose agar
VRBG:	violet red bile glucose agar

3.3 INTRODUCTION

Fishing is the second largest export industry after mining and earned about 25% of the total export value for Namibia in 2002 (Meyn, 2005). Of these exports hake constituted about 45% of the total export value of the Namibian fishing industry by 1998 (Ministry of Fisheries and Marine Resources, 2004). Hake is initially processed off shore where the head and intestines are removed on board of vessels and the fish is kept frozen for 7-8 days before being delivered for processing into fillets at the land based facilities. At the processing plant the fish is first defrosted before being sliced into fillets by the filleting machine (Baader, Germany) followed by trimming of the fillets and rinsing with water before final packaging and freezing for export. All exported fish including hake is subjected to microbiological tests to ensure compliance with the EU Directive EU 91/493/EEC (Iyambo, 1995) in order to ensure both the quality and safety of the product. As part of the evaluation total viable counts (TVC), total coliforms, faecal coliforms, *Vibrio* species, *Staphylococcus aureus* and *Escherichia coli* levels have to be determined.

Concerns have been raised by the fishing industry with regard to the microbial quality of hake since premature spoilage and fillets loosing their firmness have been noted. Deterioration of the processed product is part of a global problem that causes huge economic losses (Gram and Dalgaard, 2002; Huis in't Veld, 1996). Although spoilage could be due to endogenous enzymes (Chang et al., 1998, Chytiri et al., 2004; Ordóňez et al., 2000), it is widely found that bacteria play an important role (Gennari et al., 1999; Gram, 1992; Tryfinopoulon et al., 2002). It was reported that the number and identity of the initial fish microflora and those present after processing play an important role in determining the shelf life of the fish (Gram and Huss, 1996; Koutsoumanis and Nychas, 1999). In temperate regions fish spoilage is caused by a range of Gram negative bacteria including *Shewanella putrefaciens*, *Photobacterium phosphoreum, Pseudomonas*, *Aeromonas*, *Acinetobacters*, *Psychrobacter*, *Flavobacterium*, and *Brochothrix thermosphacta* (Chytiri et al., 2004; Gennari et al., 1999; Tryfinopoulon et al., 2002). *Moraxella*, *Corynebacterium*, *Pseudomonas, Micrococcus* and *Shewanella* predominate

under cold storage in seafood harvested from both temperate and tropical regions (Gram and Huss, 1996; Ordóñez et al., 2000).

Another concern for the producers and export countries alike is the safety of the hake harvested from Namibia. Questions with regard to the presence of pathogens such as pathogenic *Vibrio* species have been raised. Should *V. cholerae* or any of the other pathogenic *Vibrio* spp. (*V. parahaemolyticus*, and *V. vulnificus*) be detected on the hake by importing countries they will reject the whole consignment that will result in huge economic losses to the fishing industry. Human vibriosis is typically acquired through consumption of contaminated seafood. These pathogens may be present due to either the ubiquity of the causative agents in aquatic environments (DePaola et al., 2003; Harriague et al., 2008) or through contamination during processing.

The aim of this study was to determine and highlight potential factors that may lead to the deterioration of the microbial quality of hake during processing and impact on the safety of the final product. The microbiological quality of the hake was assessed at three sampling points during processing. Apart from the incoming fish, fillets after processing by the filleting machine (ABM-S) and the finished product after packaging (FP) were also sampled. The filleting machine was targeted as a possible source of contamination due to its compact mechanical nature that could prove difficult during cleaning and sanitation. The initial samples were taken at the time when sea-water was used for washing the fish during filleting. A second limited sampling programme was performed later when fresh water was used for the same purpose. The results from these two sampling periods were also compared.

3.4 MATERIALS AND METHODS

3.4.1 Sampling

During the first sampling period, hake samples were taken from three points along the processing line. These samples included the headed and gutted fish kept on ice (H and G) after delivery from the fishing boat, fillets after processing by the filleting machine (ABM) and the finished product after packaging and ready for freezing (FP). All samples were collected in food sampling bags, transported to the laboratory and either analysed immediately or kept frozen at -20°C until analysed within 24 hours. Frozen samples were thawed into the refrigerator at 2-5°C for not more than 18 hours (ICMSF, 1978a).

During the first sampling period, treated sea-water was used for defrosting incoming fish and the washing of filleted fish at filleting. A total of 120 hake samples were analysed; 20 headed and gutted fish, 20 samples after the filleting machine (ABM-S), and 60 finished products (FP). The FP sampled were either the laminated and folded hake fillets (LFHF), fillets with the skin on or the Mascato packs. The samples consisted either of a whole fish or fillets with a weight of about 300 g. The sample descriptions and number of samples are summarized in Table 3.1. During a smaller follow-up study twenty (20) fillets were re-sampled at the ABM point. At this time only fresh water was used for washing of the fillets after they were filleted (ABM-F). Each sample was analysed in five replicates.

3.4.2 Mesophilic and psychrotrophic plate counts

The total aerobic plate count was performed according to the method described by ICMSF (1978a) and Kaysner et al. (1992) for psychrotrophic and mesophilic counts respectively. A resuscitation step was included to aid recovery of potentially stressed or damaged cells including those present in the frozen samples. For resuscitation 25 g of fish tissues were transferred to a stomacher bag containing 225 mℓ of 0.1% peptone water

(PW) (Oxoid) and hand minced for one minute at room temperature (22°C) to obtain a homogenous suspension. Serial decimal dilutions of homogenates were prepared up to 10^{-6} and plated on plate count agar (PCA) (Oxoid) and Sea Water Agar (SWA) (Farmer and Hickman-Brenner, 1991) prepared using natural purified sea-water in place of artificial sea-water. Plates were incubated at 35°C for 24 h (mesophilic count) and 22°C for 72 h (psychrotrophic count) respectively. All colonies were counted.

3.4.3 Sucrose fermenting *Vibrio* species

Tissues (25 g) were aseptically excised, minced in 225 mℓ alkaline peptone water (APW) pH 8.4 (ICMSF, 1978a ; Kaysner et al., 1992) and incubated at 22°C for 6 hours according to Farmer and Hickman-Brenner, (1991) to aid in the recovery of any damaged cells. Serial decimal dilutions of the homogenates were prepared in APW up to 10^{-3}. One hundred µℓ aliquots of each dilution were spread in duplicate on Thiosulphate Citrate Bile salts Sucrose (TCBS) agar plates (Oxoid), and incubated at 35°C for 24 hour (ICMSF, 1978a; Kaysner et al., 1992). Colonies that appeared on TCBS agar as large, smooth and yellow, with flattened centres and translucent peripheries were counted.

3.4.4 Sucrose non-fermenting *Vibrio* species

Fifty grams (50 g) of fish tissue, were aseptically excised, hand minced in 450 mℓ 3% NaCl (Kaysner et al., 1992). Serial decimal dilutions were prepared in 3% NaCl up to 10^{-4}. Aliquots of the serial dilutions (10 mℓ) were inoculated into 10 mℓ double strength glucose salt teepol broth (GSTB) (Kaysner et al., 1992) and incubated at 35°C for 6 hours. Aliquots of the serial dilutions in GSTB (0.1 mℓ) were surface plated onto TCBS (Oxoid), and incubated at 35°C for 24 h. Round, bluish or green colonies 2-3 mm in diameter (Arias et al., 1998; Kaysner et al., 1992) were recorded.

Table3.1 Fish samples analysed

Sample	Description	Number of samples analysed
H and G	Headed and gutted fish stored on ice	20
ABM-S	Filets taken after filleting using sea water	20
ABM-F	Filets taken after filleting using Fresh water	20
FP (finished product)	Laminated, folded hake Fillets	20
	Fillets with skin on	20
	Mascato packs	20
Total Samples analysed		120

3.4.5 *Enterobacteriaceae*

Fish tissue (10 g) was aseptically excised, mixed with 90 mℓ of buffered peptone water (BPW) in sterile polythene bags and hand minced. The samples were decimally diluted in series up to 10^{-4} in BPW. After thoroughly mixing, dilutions were incubated at 35°C for 6 hours (ICMSF, 1978b). Aliquots (1mℓ) of the dilutions were transferred in duplicates to sterile 90 mℓ Petri dishes. Fifteen mℓ of cooled violet red bile glucose (VRBG) agar (Oxoid) was added and immediately mixed with the sample. After the agar had set a second layer (10 mℓ) of VRBG agar was added, allowed to set and the plates were incubated at 35°C for 24 hours (Chouliara et al., 2004; Paleologos et al., 2004) after which the number of pink colonies were recorded.

3.4.6 *Pseudomonas, Shewanella putrefaciens* and *Aeromonas*

For these analyses 25 g of fish tissue were placed in sterile stomacher bags containing 225 mℓ basal medium (BM) (Baumann and Baumann, 1991) and hand minced.

Homogenates were incubated at 22°C for 6 hours to aid in the recovery of any damaged cells. Thereafter serial dilutions were made in BM up to 10^{-4}. Aliquots (0.1 mℓ) of each dilution were transferred to cetrimide fusidin cephaloridin agar (CFC) (Oxoid) supplemented with supplement SR 103, (Oxoid) for culturing potential *Pseudomonads* (Chouliara et al., 2004; Chytiri et al., 2004; Paleologos et al*.,* 2004). The CFC plates were incubated at 20°C for 2 days. Small grey round colonies on CFC were scored as *Pseudomonas* spp.*.*

For *Shewanella putrefaciens*, 1 mℓ of the same dilutions was added to 10 mℓ of molten (45°C) iron agar (IA) (Oxoid), poured into 90 mℓ Petri plates and allowed to set. After setting a further 10 mℓ of IA was added as a seal, allowed to set and the plates were then incubated at 20°C for 4 days. Black colonies forming on IA were scored as presumptive *Shewanella putrefaciens* colonies (Chytiri et al., 2004).

For *Aeromonas* 0.1 mℓ aliquots of the dilutions enriched in APW as described for *Vibrio* species enrichment were spread in duplicate onto *Aeromonas* agar (Oxoid) to which supplement SR 151 (Oxoid) was added. Plates were incubated at 25°C for 48 hours according to Farmer et al. (1991). Pale green colonies were scored as presumptive *Aeromonas* spp. Average logs values from counts of presumptive *Pseudomonas*, *Shewanella* and *Aeromonas* were calculated after 5 replicate samples were processed.

3.4.7 Statistical analysis

A number of statistical methods were used. In order to be able to compare the data from the different stages during the processing, average log values of counts were first determined for all the microbial analyses and the standard deviations were calculated using Microsoft excel. ANOVA for Randomised complete Block Design and the least significant difference technique to separate means was also used. In some instances the nested hierarchal approach was used in cases where the factors were nested.
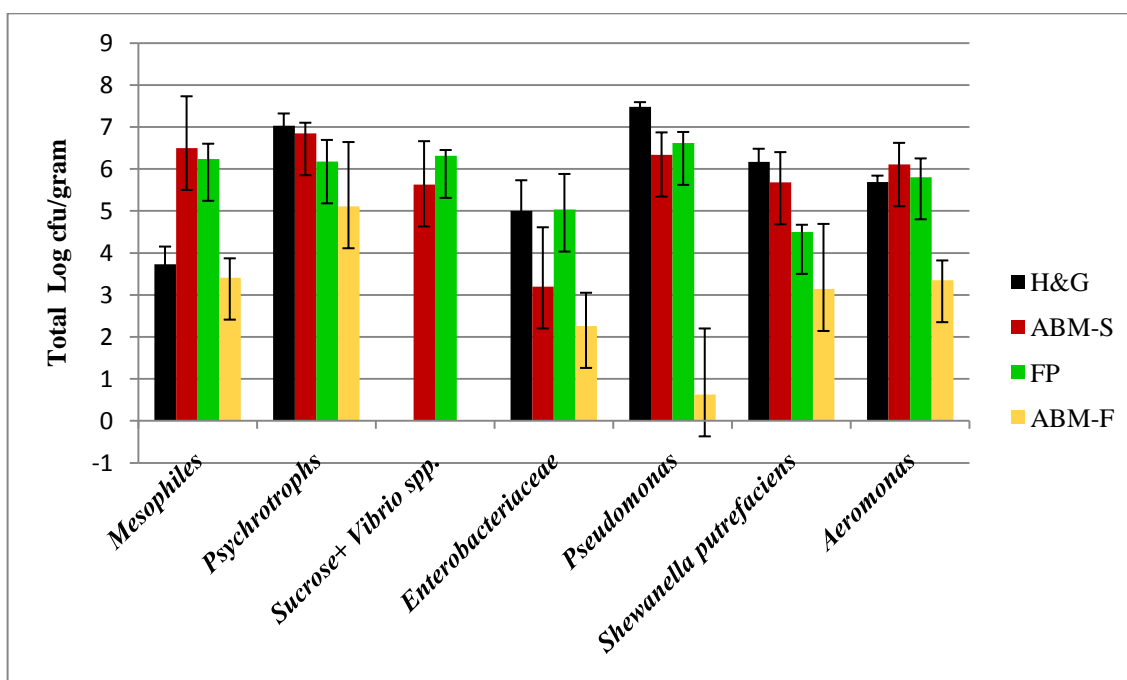
3.5 RESULTS

The microbial data for the sampling period when sea-water was used in the processing plant are shown in Figure 3.1. At the beginning of the processing line (H and G) the mean mesophilic aerobic plate count was log 3.73 cfu/g, increasing to log 6.50 cfu/g and 6.24 cfu/g after the filleting machine (ABM-S) and in the final products (FP) respectively. The increase in mesophilic count by nearly 3 logs along the processing line suggests either loss of temperature control or exogenous contamination along the processing line. The mean psychrotrophic aerobic plate count remained fairly constant during processing (Figure 3.1). The measured count for the incoming fish (H and G) was log 7.03 cfu/g. At ABM-S and FP the recorded psychrotrophic counts were log 6.85 cfu/g and 6 18 cfu/g respectively.

No bacteria grew on TCBS after APW enrichment of the incoming fish (H and G). At the filleting machine (ABM-S) and in the final product the level of sucrose fermenting bacteria rose sharply with an average log of 5.63 cfu/g and 6.61 cfu/g respectively when treated sea water was used for processing. This increase in potential *Vibrio* levels indicated exposure of hake to some form of contamination during processing. Sucrose fermenting *Vibrio* species were not detected when fresh water was used (Figure 3.1). No non-sucrose fermenting bacteria with characteristic appearance of *Vibrio parahaemolyticus* were detected in any of the samples.

Enterobacteriaceae counts on the fish were the same at the beginning (H and G) and at the end of the processing line (FP) with levels of log 5.00 cfu/g and 5.03 cfu/g respectively. A lower value of log 3.20 cfu/g was noted at the intermediate stage (ABM-S). High counts of *Pseudomonas,* similar to those observed for the psychrotrophic counts, were observed throughout processing. The mean levels were log 7.48 cfu/g (H and G), log 6.34 (ABM-S) and log 6.62 cfu/g (FP). The *S. putrefaciens* counts decreased during processing and were log 6.17 cfu/g (H and G), log 5.68 cfu/g (ABM-S) and log 4.5cfu/g

(FP) respectively. The *Aeromonas* levels remained fairly constant at log 5.69 cfu/g (H and G), log 6.11 cfu/g (ABM-S) and log 5.8 cfu/g (FP).

As the use of treated sea-water to wash the fish after filleting was discontinued another set of samples were taken. In most cases the bacterial levels differed significantly from those measured during the first sampling period (Figure 3.1). The mean mesophilic count recorded was log 3.41 cfu/g, 3 logs lower than the previous count obtained when sea-water was used at this point. The count of *Enterobacteriaceae* was log 2.26 cfu/g, that of *Pseudomonads* log 0.63 cfu/g, *S. putrefaciens* log 3.14 cfu/g and *Aeromonas* log 3.35cfu/g. All of these results were significantly different from the original levels (p=0.001).

Figure 3.1 Total viable bacterial counts on hake. H and G= Headed and gutted, ABM-S= after filleting machine with sea water, FP= Finished packed hake fish.

3.6 DISCUSSION

The microbial quality of processed fish is usually determined by a number of factors, including the levels of microbes on the raw product, the microbial contamination during processing and the exposure of the product to conditions that will allow for the multiplication of the existing microbes on the product. High mesophilic counts in marine fish are usually indicative of the existence of such conditions and may signal a potential spoilage or health hazard as many spoilage and pathogenic bacteria are mesophilic (ICMSF, 1978a). Total viable aerobic counts on seafood are normally in the ranges of (ca. 10^4 -10^6cfu/g) on the skin, (10^4-10^7 cfu/g) in the gills (Gennari et al., 1999), and (10^4-10^6cfu/g) in the intestines (Austin and Austin, 1987).

In this study the mean viable mesophilic count was log 3.73 cfu/g on the raw product (H and G) but increased dramatically to a value of higher than log 6 cfu/g at the intermediary stage (ABM-S) and in the finished product (FP). The levels determined for the headed and gutted fish, kept on ice on board of the fishing vessels for several days before the fish is delivered to the factory, compared well with other studies (Cakli et al., 2006; Pastoriza et al., 1996; Tzikas et al., 2007).

The mean psychrotrophic counts obtained in this study remained high at nearly the same level during all the stages of processing. High psychrotrophic counts observed could have originated from the natural flora of hake that multiplied from the time of the fish catch to the time of delivery to the factory indicating potential problems on board of the fishing vessels. The psychrotrophic counts are usually representative of normal spoilage organisms such as *Pseudomonas* and *Shewanella* spp. (Gram and Dalgaard, 2002) that can grow at refrigeration and ambient temperatures. This was confirmed by Ordóňez, et al. (2000) who also showed that *Pseudomonas* and *Shewanella* were the predominant spoilage bacteria on gutted hake stored on ice. In this study the *Pseudomonas* counts of log 7.48cfu/g in the incoming fish and log 6.62 cfu/g in the final hake products, were

similar to those measured for the total psychrotrophic counts. These values may indicate a short product shelf life.

Aeromonas spp. could also form part of the psychrotrophic bacteria and have been isolated from a number of marine and fresh water species fish (Papadopoulou et al., 2007). They are also fish spoilage organisms and may produce H_2S. In this study counts ranged from log 5 to log 6 cfu/g and hake spoilage due to this group of bacteria can therefore not be excluded.

S. putrefaciens is typically one of the predominant microflora of ice stored fish from temperate regions (Chytiri et al., 2004; Gennari et al., 1999; Paarup et al., 2002). In this study the levels of sulphate reducing bacteria (SRB) including *S. putrefaciens* in H and G and FP hake were log 6.17 and log 4.50cfu/g respectively. Some reduction in the levels of *S. putrefaciens* was noted as the fish moved along the processing line. Despite this reduction the levels are still of concern and it should be noted that the method of keeping fish on board for several days before delivery to the factory for final processing may have a negative effect on microbiological quality and could lead to spoilage.

Some of the counts were higher than what was reported in literature (Vennemann, 1991; Tsikas et al., 2007). This could be linked to a recovery step that was included in the analysis of some of our samples. This step was included as Tsikas et al. (2007) have observed a lag phase in the growth of total viable bacteria performed on Mediterranean horse mackerel and blue jack mackerel muscle done after 4 to 6 days of fish storage on ice. Within fish processing environments bacteria are also continually exposed to stressing situations such as chill temperatures, and the presence of sanitizers that cause sublethal injury to bacteria. An enrichment step often assists with the recovery of these bacteria (Foegeding and Ray, 1992; ICMSF, 1978b)

Human pathogens are typically mesophilic bacteria with an optimum growth range between 30-45°C (Forsythe and Hayes, 1998). An increase in the mesophilic count is therefore of potential health concern. *Enterobacteriaceae* are widely distributed in

aquatic environments including marine waters (Papadopoulou et al., 2007) and could be one of the reasons for the observed increase. High counts of *Enterobacteriaceae* typically indicate potential faecal contamination (ICMSF, 1978a). During this study the *Enterobacteriaceae* initial counts for hake were log 5.00 cfu/g (H and G), and similar count was observed in the finished products. These counts were similar to those obtained by Ordóñez et al. (2000) on hake steaks before storage, and by Economou et al. (2007) in tuna fish which was kept at 20°C. Himelbloom et al. (1991) found lower (10^2 cfu/g) *E. coli* counts on Alaskan finfish. In this study it was therefore demonstrated that the levels were within the expectable norms, and faecal contamination of the processed fish was not suspected to be the reason for the deterioration in the mesophilic counts.

The sharp increase in mesophilic counts observed during processing was still a cause for concern as this trend corresponded with a similar increase in the levels of sucrose fermenting *Vibrio* spp. No sucrose fermenting *Vibrio* species were detected in the incoming hake (H and G), but this group of bacteria suddenly appeared at high levels in the ABM-S and FP samples. Further investigations indicated that this sharp increase in potential *Vibrio* spp. could be a result of the introduction of the bacteria by means of the treated sea-water used during processing. There were indications that the major source of these bacteria was not inefficient treatment of the raw water but the subsequent formation of biofilms in the distribution network in spite of the presence of residual chlorine (Shikongo-Nambabi et al., 2010a). Identification of the isolated sucrose fermenting bacteria confirmed that these strains were not *V. cholerae* but *V. alginolyticus* and that they did not pose any immediate health risk to any of the consumers (Shikongo-Nambabi et al., 2010b)

The impact of sea-water was further investigated when the factory was refurbished and started to use fresh water as the major source of water during processing. No sucrose fermenting *Vibrio* species were detected in any of the products tested. The differences observed for all the microbial parameters were of statistical significance indicating a positive impact on the overall quality of fish. This improvement should significantly increase the shelf life as well as the safety of the hake processed in the plant.

3.7 CONCLUSION

The microbial quality as observed during the initial study period raised a number of concerns and warranted a closer investigation to ascertain that good manufacturing practises are strictly adhered to from the time the fish is caught up to the point of processing of the final product. The results indicated that some deterioration in quality during processing could be due to contamination during processing while others may have originated with the fish supplied to the plant since all fish samples analysed during this study were not freshly caught.

This study has shown that *Pseudomonas*, *S. putrefaciens* and *Aeromonas*, and typical spoilage organisms, form part of the bacterial population on the hake. *Pseudomonas* and *Aeromonas* were present at the same level while *S. putrefaciens* levels were slightly lower. The results indicated that these organisms already formed part of the incoming fish and that process did not increase their levels dramatically. It is highly likely that the method of keeping fish on board the fishing vessels for several days before delivery to the factory for final processing may have a negative effect on microbiological quality and could lead to spoilage.

Comparison of viable bacterial counts obtained from the three stages along the processing line has revealed higher mesophilic counts in hake after filleting. Of particular interest was the sucrose fermenting *Vibrio* species that were not detected in the incoming (H and G) fish, but were detected in high numbers ca. log 6.31 cfu/g when sea-water was used to wash the hake fillets before trimming and packaging. A link was made to the treated sea -water used during processing as the most likely source of contamination. This was confirmed when a significant improvement was observed when fresh water was used to wash fish at the same point during processing. Although sea-water could be an alternative source of water for marine fish processing plants, the treatment and the quality of the water needs to be carefully managed.

3.8 ACKNOWLEDGEMENTS

3.9 REFERENCES

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CHAPTER 4

<u>SHIKONGO-NAMBABI MNNN, CHIMWAMUROMBE PM and VENTER SN (2010)</u> **IDENTIFICATION OF *VIBRIO SPP.* ISOLATES OBTAINED FROM PROCESSED MARINE FISH.** *Proceedings of the National Symposium 15-17 September Safari Hotel Windhoek, Namibia* (In print)

4.1 ABSTRACT

Vibrio species are ubiquitous in marine environments and contaminate marine fish during processing. *V. cholerae*, *V. parahaemolyticus, V. vulnificus* are human pathogens causing cholera, gastroenteritis, septicaemia and wound infections that can be fatal in humans. *V. alginolyticus* is an opportunistic pathogen and maybe of less importance. The presence of these bacteria in fish could pose serious health risks to consumers and creates trade barriers for Namibia. The aim of this study was to identify 243 Gram negative bacteria isolated from hake, pilchards and horse mackerel during processing using Thiosulphate Citrate Bile salts Sucrose (TCBS) agar. Initially basic phenotypic tests such as oxidase and O-F tests were done. This indicated that 92 (38%)of the isolates were typical *Vibrionaceae.* Secondly the isolates were screened for genes specific to the human pathogenic *Vibrio* species by Polymerase Chain Reaction (PCR) targeting the *ompW* of *V. cholerae*, *pR72H* fragment of *V. parahaemolyticus* and the cytolysin-haemolysin gene of *V. vulnificus*. This was followed by sequencing the 16S rRNA gene and drawing Neighbour Joining phylogenetic trees based on the data. All suspect isolates were further characterised by a combination of selected phenotypic tests and the *V. alginolyticus* specific collagenase gene PCR. None of the bacteria isolated from hake, pilchard or horse mackerel were identified as *V. cholerae, V. parahaemolyticus* or *V. vulnificus*. Out of the 80 isolates that were identified as *Vibrionaceae* by their 16S rRNA gene sequences sixty-three could be identified as *V. alginolyticus*. The remaining 17 isolates belonged to the genera *Listonella, Shewanella* and *Aeromonas*. The study therefore indicated that the three fish species examined do not pose an immediate health hazard to consumers. It also showed that when identifying *Vibrio* spp., particularly when it is necessary to differentiate between *V. alginolyticus* and *V. parahaemolyticus,* a polyphasic approach is still most desirable.

KEYWORDS: *V. parahaemolyticus, V. alginolyticus,* V. *vulnificus*, identification, PCR, 16S rRNA, hake, pilchards and horse mackerel.

4.2 INTRODUCTION

The family *Vibrionaceae* represents a number of genera including *Vibrio*, *Grimontia*, *Photobacterium* and *Salinivibrio* (Thompson et al., 2005). Amongst the *Vibrio* species there are at least 13 species pathogenic to humans (*V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. funissii, V. fluvialis, V. damsela, V. mimicus, V. hollisae, V. cincinnatiensis, V. harveyi, V. metchnikovii* and *V. carchariae*) causing cholera, gastroenteritis, septicaemia and wound infections (Drake et al., 2007). Many *Vibrios* are natural inhabitants of the marine environment, largely in association with fish and zooplankton (Montanari et al., 1999) and may contaminate marine fish during processing (Wekell et al., 1994). Temperature abuse often results in the uncontrolled proliferation of these bacteria in sea food and represents one of the most common routes of transmission of these pathogens to humans (Joseph et al., 1982).

The identification of *Vibrio* species is commonly done using classical phenotypic methods and 16S rRNA sequence comparisons are often included (Thompson and Swings, 2006; Drake et al., 2007). Classical techniques, however, take a long time to conduct and may sometimes be inaccurate as some strains exhibit atypical phenotypic characteristics (Oliver and Kaper, 1997). Comparison of the 16S rRNA sequences can in most cases only correctly identify *Vibrio* isolates up to genus level due to high interspecies similarities. It has, for example been difficult to correctly differentiate *V. alginolyticus* from the closely related *V. parahaemolyticus* which share between 60 -70% DNA homology (Oliver and Kaper, 1997; Farmer et al., 2004). Molecular methods targeting and amplifying unique genes with PCR have also been used, sometimes with limited success (Drake et al., 2007). We have recently reported that the microbiological quality of hake deteriorated during processing at a marine fish processing facility in Walvis Bay, Namibia. Sucrose fermenting *Vibrios* were isolated at the intermediate stage of the processing line while none were detected on the incoming fish (Shikongo-Nambabi et al., 2010a). *Vibrio cholerae* is predominantly a sucrose fermenter and accurate identification of these isolates was therefore very important. Should this or any related pathogens be present in the processed fish exported to the European

countries, the whole consignment would be returned back to Namibia as a sign of non-compliance with the trade agreement reached between Namibia and these countries. It was also noted that the isolated *Vibrio* species were able to form multicellular complexes that defy the action of oxidizing agents commonly used in the fishing industry as sanitizers (Shikongo-Nambabi et al., 2010b). Accurate identification of these isolates would help eliminating any doubt surrounding the presence of pathogenic *Vibrio* species and the safety of the Namibian hake.

During this study 247 bacteria isolated from processed fish were identified to establish whether any pathogenic *Vibrio* spp. are associated with these products. The isolates obtained during TCBS isolations (both sucrose and non sucrose fermenters) were identified by a polyphasic approach. This included phenotypic methods (Gram stain reaction, oxidase, and O-F tests), amplification of unique genes and sequencing of the 16S rDNA. One hundred and eighty-four of the isolates were obtained from processed hake, 27 isolates were from pilchards and 15 from horse mackerel (HM). Many of the isolates gave conflicting reactions to different identification methods and could only be identified once the collective data were analysed.

4.3 **MATERIALS AND METHODS.**

4.3.1 Bacterial isolates

All 247 isolates, as previously described in Chapter 3, were obtained from hake, pilchard, or horse mackerel. Samples were plated on Thiosulphate Citrate Bile salts Sucrose (TCBS) agar after pre-enrichment in either (Alkaline peptone water) APW pH 8.6 or in 3% NaCl-Glucose Salt Teepol Broth (GSTB). Apart from the 247 isolates obtained from the fish, four reference strains were also included in the study to serve of positive controls. *Vibrio cholerae* isolate (C453) was kindly supplied by Prof. SN Venter, Department of Microbiology and Plant Pathology, University of Pretoria. Type strains of *Vibrio parahaemolyticus* (LMG 2850), *V. vulnificus* (LMG13545) and *V. alginolyticus* (LMG 4409) were obtained from the Belgium Culture Collection, Laboratorium voor Microbiologie, Ghent (BCCM/LMG) University of Ghent, Belgium.

4.3.2 The Gram stain differentiation

A single colony from an overnight pure culture was suspended in 50 μl of 10% KOH to determine the Gram reaction of the isolates. Cultures that showed an increase in viscosity in the suspension and the formation of a characteristic slimy string were recorded as being Gram negative. If no string was seen the culture was recorded as Gram positive (Buck, 1982). A *Micrococcus* spp. isolate was used as a negative control.

4.3.3 The Oxidase test

The oxidase test was performed according to Quin et al. (1994). The colony to be tested was picked with a sterile toothpick and streaked across filter paper placed in a Petri dish. The filter paper was flooded with a freshly prepared 1% (w/v) Oxidase (N-N-N-N-Tetra methyl phenylene diamine dihydrochloride) solution. The test was recorded as positive

when the cells turned dark purple within 5-10 seconds. *Vibrio cholerae* isolate (C453) was used as a positive control.

4.3.4 The Oxidation/Fermentation (O/F) Test

The ability of isolates to utilize glucose under both aerobic and anaerobic conditions was tested with O-F medium (Biolab). The semisolid medium (1%) was prepared according to the manufacturer's instructions. The medium was sterilised by autoclaving at $121^{\circ}C$ for 20 minutes. As carbon source, 10 mℓ of a filter sterilized 10% D-Glucose solution was added to the autoclaved medium. The medium (5 mℓ) was dispensed into sterile test tubes. For each test culture two tubes were prepared, the anaerobic tube was overlaid with 1 mℓ sterile mineral oil. The test was performed by stab inoculating the medium and then incubating it at $37^{\circ}C$ for 24 hours. When both tubes turned yellow the isolate was taken as being facultative anaerobic or fermentative. When only the tube without oil turned yellow, the isolate was regarded as aerobic or oxidative. If none of the test tubes turned yellow the isolate was regarded as glucose inactive (Quin et al., 1994). *Vibrio cholerae* isolate (C453) was used as a positive control.

4.3.5 Polymerase Chain Reaction (PCR)

A total of 247 cultures suspected to be *Vibrio* species by their growth on TCBS agar, 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* (LMG13545) and *V. cholerae* (C453) were included as positive controls. Isolates that reacted in *V. parahaemolyticus* specific PCR were screened for the species specific virulence genes, thermostable direct haemolysin (*tdh*) and thermostable direct haemolysin related haemolysin (*trh*). All isolates that reacted with any of the pathogen specific PCR tests were subjected to 16S rDNA sequencing (Coenye et al., 1999).

4.3.5.1 Chromosomal DNA extraction

DNA required for PCR or sequencing was extracted as described by Le Roux et al. (2004). Bacterial cultures (either test or reference strains) were grown overnight on Nutrient Agar supplemented with 3% sodium chloride (NaCl) or on sea water agar (SWA) (Atlas 2006; Farmer and Hickmann-Brenner, 1991). DNA was extracted by suspending a loopful of the culture in 500 µℓ of sterile distilled water (SDW). After vortex mixing to obtain a homogenous suspension, the cells were boiled for 10 minutes. Cell debris was removed by centrifugation at 1000 rpm for 60 seconds. The supernatant (200 µℓ) was transferred to a sterile 1.5 mℓ Eppendorf tube and frozen at -20°C till used. In some cases DNA was extracted with the Qiagen DNA extraction kit according to the manufacturer's instructions.

4.3.5.2 Oligonucleotide Primers

Oligonucleotide primers used to amplify species specific genes and pathogenicity factors or the sequencing of the16S rRNA are listed in Table 4.1. All primers were synthesised by Inqaba Biotechnology, Pretoria, Republic of South Africa (RSA).

4.3.5.3 PCR conditions

The *V. cholerae* targeting primers (VIB1 and VIB3) were used to directly amplify a 305 bp fragment of the *ompW* gene (Nandi et al., 2000). The PRC was carried out in a 20 µℓ reaction mixture containing 2.0 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 5.0 µℓ of template DNA. The rest of the volume was filled with nuclease free water. The PCR cycle consisted of an initial denaturation step at 94°C for 3 minutes, final strand extension at 72°C for 7 minutes and 30 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minute and DNA extension at 72°C for 1 minute.

For the *Vibrio parahaemolyticusp R72H* fragment, PCR reactions were carried out in 30 µℓ reaction mixtures consisting of 3.0 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. The PCR cycle consisted of an initial denaturation step at 94^oC for 5 minutes, and a final extension at 72^oC for 7 minutes, with 35 cycles each consisting of denaturation at 94^oC for 1 minute, primer annealing at 60^oC for 1 minute, and primer extension at 72^oC for 1 minute (Lee et al., 1995; Robert-Pillot et al., 2002).

Detection of *V. parahaemolyticus* virulence genes, *tdh* and *trh* was done in a reaction mixture consisting of 5.0 µℓ 10 × PCR Buffer, 20.0 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 consisted of an initial denaturation step of 94^oC for 5 minutes, final extension at 72^oC for 5 minutes, thirty cycles, each consisting of denaturation at 94^oC for 1 minute, primer annealing at 58^oC for 1 minute, and primer extension at 72^oC for 1 minute (Bej et al., 1999).

For the amplification of the *V. vulnificus* cytolysin-haemolysin gene, the PCR was performed in a 50 µℓ reaction mixture consisting of 10 µℓ of 10 × PCR Buffer, 40.0 µM dNTP's (Thermo Scientific), 2.0 µM of $MgCl_2$, 1.0 µM of each of the primers, (*Choi-1* and *Choi-2*), 0.15 IU/µℓ DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology), 2.0 µℓ of test DNA and nuclease free water to the final volume. The PCR cycle consisted of an initial denaturation at 94^oC for 3 minutes, and final primer extension at 72^oC for 10 minute with 45 cycles each consisting of denaturation, at 94^oC for 1minute, primer annealing at 60^oC for 1 minutes and primer extension at 72^oC for 2 minutes (Lee et al., 1997).

Table 4.1 Primer sequences and their associated gene targets

Primers	Target gene and organism	Amplicon Size (bp)	Primers Sequence (5' to 3')	Reference
VIB1 (Forward)	*ompW* *V. cholerae*	305	CACCAAGAAGGTGACTTTAATTGTG	Nandi et al., (2000)
VIB3 (Reverse)			GGTTTGTCGAATTAGCTTCACC	Nandi et al., (2000)
Vp32 (Forward)	pR72H *V. parahaemolyticus*	387 or 320	TGCG AATTCGATAGGGTGTTAACC	Lee et al., (1995);
Vp33 (Reverse)			CGAATCCTTGAACATACGCAGC	Robert-Pillot et al., (2002)
Choi1 (Forward)	Cytolysin-haemolysin *V. vulnificus*	704	GACTATCGCATCAACAACCG	Lee and Choi (1995)
Choi2 (Reverse)			AGGCGAGTATTACTGCC-3'GTA	Lee and Choi (1995)
pA (16F27)	16SrRNA of prokaryotes	1458	AGA GTT TGA TCC TGG CTC AG	Coenye et al., (1999)
MH2 (16R1485)			TAC CTT GTT ACG ACT TCA CCC CA	Coenye et al., (1999)
*pD (16F536)	16S rRNA sequencing	849	CAGCAGCCGCGGTAATAC	Coenye et al., (1999)
VA-F (Forward)	Collagenase *V. alginolyticus*	737	CGAGTACAGTCACTTGAAAGC	Di Pinto et al., (2006)
VA-R (Reverse)			CACAACAGAACTCGCGTTACC	Di Pinto et al., (2006)
L-trh (Forward)	*TDH-related haemolysin of V. parahaemolyticus*	500	TTGGCTTCGATATTTTCAGTATCT	Bej et al., (1999)
R-trh (Reverse)			CATAACAAACATATGCCCATTTCC	Bej et al., (1999)
L-tdh (Forward)	*Thermostable direct haemolysin of V. parahaemolyticus*		GTAAAGGTCTCTGACTTTTGGAC	Bej et al., (1999)
R-tdh (Reverse)		269	TGGAATAGAACCTTCATCTTCACC	Bej et al., (1999)

For the *V. alginolyticus* collagenase gene, PCR amplification of the gene was carried out in a reaction mixture consisting of 5.0 µℓ 10 × PCR Buffer, 20.0 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, 3.0 µℓ of DNA. The volume was made up to 50 µℓ with nuclease free water. The PCR cycle consisted of initial denaturation at 95°C for 15 minutes, and the final primer extension at 72°C for 5 minutes, 35 cycles, each consisting of denaturation at 94°C for 30 seconds, primer annealing at 57°C for 30 seconds and primer extension at 72°C for 1 minute (Di Pinto et al., 2006).

The 16S rDNA was amplified in a total volume of 50 µℓ, consisting of 5 µℓ of 10 × PCR buffer, 2.0 µM of dNTP's (Thermo Scientific), 2.0 mM of MgCl$_2$, 5.0 µM of each primer, 0.0005 IU/µℓ of Taq DNA polymerase (Super-therm polymerase JMR, Southern Cross Biotechnology) 2.0 µℓ of template DNA and nuclease free water to make up the volume. The PCR cycle included the following steps. Initial denaturation at 94°C, a final primer extension at 72°C for 5 minutes and 30 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1minute and primer extension at 72°C for 5 minutes (Coenye et al., 1999). Bands of the desired size (1458 bp) and intensity 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 DNA was substituted for nuclease free water in the negative control. All PCR mixtures were held at 4°C 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 rDNA 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 96°C for 5 seconds, a final extension at 60°C for 4 minutes, and 25 cycles, each consisting of denaturation at 96°C for 10 seconds, primer annealing at 55°C for 5 seconds and the primer extension at 60°C for 4 minutes.

4.3.6 Sequence alignment and phylogenetic analysis.

Sequence alignments were carried out using Clustal X software (Thompson et al., 1997) and the sequences were edited and trimmed in Bioedit Sequence alignment Editor v. 5.0.9 (Hall, 1999). Phylogenetic trees of the 16S rDNA sequences were draw with PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 2000) using the Neighbour –Joining (NJ) method (Saitou and Nei, 1987). Confidence limits were determined using Bootstraps (Efron, 1979). Thirty-one sequences of known species, mainly type strains, obtained from GenBank (http://www.cnbi.nlm.nih.gov/Genbank/) were included in the phylogenetic trees to assist with the final grouping of the unknown sequences (Sneath, 1989).

4.3.7 API 20E and other biochemical tests

Due to unresolved identifications for many of the isolates based on the 16S rRNA sequencing, extra phenotypic data were collected for these isolates. The phenotypic data included the Analytical Profile Index (API) 20E in conjunction with individual biochemical tests such as cellobiose and sucrose fermentation and growth in Nutrient broth containing 10 and 12% sodium chloride (Farmer et al., 2004; Oliver and Kaper, 1997).

The API 20E (BioMérieux) was selected to assist with the identification especially to help differentiate between *V. parahaemolyticus* and *V. alginolyticus* strains (Oliver and Kaper, 1997; Farmer et al., 2004) For this purpose special attention was given to the Voges-Proskauer (VP) test, ornithine decarboxylase activity and arabinose fermentation. The API 20E test was done according to the supplier's instructions and proposed species identification was obtained using the API lab software.

4.3.8 Phenotypic comparison

The 24 phenotypic characters for 52 isolates that clustered with *Vibrios* were also imported into Bionumerics and a similarity coefficient dendrogram was drawn (Austin and Colwell, 1977) to demarcate isolates as either *V. parahaemolyticus* or *V. alginolyticus*. These characters include 20 miniaturized biochemical tests on API 20E, the cellobiose fermentation test determined according to Jones and Wise, (1925) in a sterile 1% cellobiose solution supplemented with 3% sodium chloride and 0.15% Bromocresol purple as pH indicator, sucrose fermentation test by growing the isolates on TCBS, the oxidase test as described by Quin et al. (1994) and the ability to growth in nutrient broth containing 10% sodium chloride (Farmer et al., 2004; Oliver and Kaper, 1997;). *V. parahaemolyticus* LMG 2850 and *V. alginolyticus* LMG4409 were used as controls.

4.4 RESULTS.

A total of 247 bacteria were selected from TCBS agar and subjected to a number of identification protocols; Gram reaction, oxidase and Oxidative/Fermentative (O/F) tests, PCR detection of species specific genes (*V. cholerae ompW*, *V. parahaemolyticus pR72H* fragment, and the *V. vulnificus vvh* and the *V. alginolyticus* collagenase gene), 16S rRNA gene sequence alignment, phylogenetic analysis using the 591 bp fragment of the 16S rDNA, API20E, and the numerical taxonomy using 24 phenotypic attributes.

4.4.1 Gram reaction, sucrose fermentation, oxidase and O-F tests

Two hundred and forty-three (98. 3%) isolates formed the characteristic string in the KOH test and were confirmed as Gram negative bacteria. Only four (1.6%) of the 247 isolates were negative for the KOH test and were considered to be Gram positive (Table 4.4). The four Gram positive isolates were excluded from other tests that followed since our concern lay with the Gram negative bacteria of the γ subclass of the *Proteobacteria*. Only 243 isolates will be discussed further. One hundred and twenty-eight (52.6%) of the Gram negative bacterial isolates were sucrose fermenters, while one hundred and fifteen (47.3%) were sucrose non fermenters.

Based on the oxidase and O-F tests performed on 243 isolates only 81 (33.3%) of the isolates were oxidase positive, fermentative or facultative anaerobic (F/O-F) hence belonging to the family *Vibrionaceae* (including genera such as *Vibrio*, *Aeromonas* and *Shewanella*). Eight (3.31%) of the isolates were oxidase positive, oxidative and possibly belonging to the family *Pseudomonadaceae*. Fifty-three (21.8%) isolates were oxidase positive but glucose inactive hence regarded as either *Vibrionaceae* or *Pseudomonadaceae*. Four (1.6%) isolates were oxidase negative and glucose inactive and were suspected to be *Enterobacteriaceae*. Eighty-four (34.6%) of the isolates were oxidase negative facultative anaerobic and were grouped into the family *Enterobacteriaceae*, of the genera such as *Morganella, Pectobacterium, Citrobacter, Providencia,* and *Proteus*. Thirteen (5.3%) of the isolates were oxidase positive, but gave doubtful results in the O-F test (Figure 4.1).

Twenty-eight (11.5%) of the 243 bacteria were isolated from headed and gutted (H and G) hake fish, fifty-eight (23.9%) were from hake fish after the filleting stage (ABM-S), ninety-six (39.5%) were from hake fish packaged products (FP), twenty-one (8.6%) were isolated from the headed and gutted pilchards Jitney (tall line) (TL), twenty-five (10.3%) were isolated from washed and packaged pilchards bait line (BL) and fifteen (6.2%) were isolated from headed and gutted demersal horse mackerel (HM).

4.4.2 Identification of isolates to species level

Due to the unusual phenotypic variability of the bacteria isolated from TCBS 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 (Colwell, 1970; Molitoris et al., 1985) a polyphasic taxonomy approach of identifying the isolates (Oosthuizen, 1998) was taken employing both genotypic and phenotypic traits. Screening of all the isolates for the presence of genes typically associated with the human pathogenic *Vibrio spp*. was used as the starting point.

4.4.2.1 Detection of species specific genes using PCR.

None of the 243 cultures screened were positive for the amplification of the 305 bp fragment of the *V. cholerae* ompW gene. Fifty-seven (23.5 %) isolates reacted with the Vp32 / Vp33 primer pair specific for the *pR72H* fragment of *V. parahaemolyticus*. Thirty three of these isolates produced two bands, 387 and 400 bp in size while 24 produced the expected single band of 387 bp (Table 4.2).

Eighty-eight (36.2%) of all the isolates reacted with the *V. vulnificus* specific Choi1 / Choi2 primer pair targeting the cytolysin/haemolysin gene, 19 of these isolates also reacted with the *V. parahaemolyticus* specific primers (Figure 4.2). 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 strain *V. vulnificus* LMG 13545. Twenty-four isolates

formed bands smaller than that of the positive control and eleven formed a band larger than the typical 704 bp product. Another sixteen isolates formed bands either equal and greater or equal and smaller to the band produced by the positive control (Table 4.2).

4.4.2.2 PCR for detection of *V. parahaemolyticus* virulence genes

Only 7 (12.1%) of the 57 isolates that were positive for the *pR72H* fragment produced a product for the L-*trh* / R-*trh* primer pair. Strong bands were detected although these bands were about 550bp and 600bp in size (results not shown).The amplified products were all of higher molecular weight than the typical 500 bp product obtained from the *V. parahaemolyticus* strain LMG2850. None of the isolates reacted with the R-*tdh*/L-*tdh* primer pair.

4.4.2.3 16S rRNA gene sequence analysis

The partial 16S rRNA genes of 119 of the 127 isolates that reacted with either the Vp32/Vp33 and/or the Choi1/Choi2 primers pairs were sequenced. Eight isolates were not sequenced because they were lost during the sub culturing process.

Table 4.2 Grouping of isolates that reacted with *V. parahaemolyticus* and *V. vulnificus* specific (*VP32/Vp33* and *Choi1/Choi2*) primer pairs according to genera

Amplification of species specific genes	*pR72H* (bp)	*vvh* (bp	Isolate/s	Number	Genera
	387	704	381, 577, 622	3	*Vibrio, Shewanella*
	387 + 400	704	445, 447, 482, 523, 524	5	*Vibrio*
	387	<704	412, 422, 430	3	*Vibrio*
	387 +400	<704	414, 415, 462, 464, 483, 522	6	*Vibrio*
	387	>704	406, 624	2	*Vibrio*
	387	-ve	382, 383, 425, 432, 435, 499, 526, 588, 590, 601, 602 606, 608, 614, 617, 619, 634,	17	*Vibrio, Shewanella*
	387 + 400	-ve	386, 388, 389, 407, 409, 410, 411, 421, 423, 426, 427, 434, 436, 439, 440, 510, 511, 517, 595, 611, 638, 645	22	*Vibrio, Citrobacter, Shewanella*

Table 4.2 continued

Amplification of species specific genes	*pR72H* (bp)	*vvh* (bp	Isolate/s	Number	Genera
	-ve	704	343, 372, 374, 377, 391, 394, 398, 408, 416, 420, 424, 428, 429, 449, 463, 465, 472, 479, 480, 481, 486, 487, 490, 525, 578, 589, 598, 599, 633, 640	29	*Aeromonas, Citrobacter, Enterobacter, Morganella, Providencia, Shewanella, Vibrio,*
	-ve	<704	385, 441, 469, 477, 569, 575, 576, 593, 600, 615, 618, 623, 626, 632, 636	15	*Enterobacter, Morganella, Shewanella, Providencia, Vibrio,*
	-ve	>704	475, 478, 494, 501, 574, 583, 596, 613, 616	9	*Morganella, Providencia, Shewanella, Vibrio,*
	-ve	$\leq/\geq$ 704	341, 390, 397, 431, 437, 445, 446, 448, 459, 460, 467, 473, 476, 500, 639, 582	16	*Aeromonas Citrobacter, Morganella, Providencia, Pseudomonas, Vibrio,*
Total	58	88		127	

4.4.2.3.1 Sequence similarities

A BLASTn search of the 591 bp sequence of the 16S rRNA of 119 isolates was compared to similar sequences in Genbank database. The results indicated that these strains were closely related to a number of known bacterial species. Thirty one isolates were found to have between 97% and 100% 16S rDNA sequence homology to *V. alginolyticus* strain X56576. Twenty-two (18.5%) of the isolates'partial 16S rRNA sequence was closely aligned to *V. parahaemolyticus*, 3 (15.0%) of which had 100%, 14 (70.0%) had 99%, 2 (10.0%) had 98%, and 3 (15.0%) had 97% 16S rDNA sequence similarity with *V. parahaemolyticus* strain AF388386. Eighty-three (69.7%) of the isolates were identified by the BLASTn as belonging to the family *Vibrionaceae* and of these isolates sixty-six
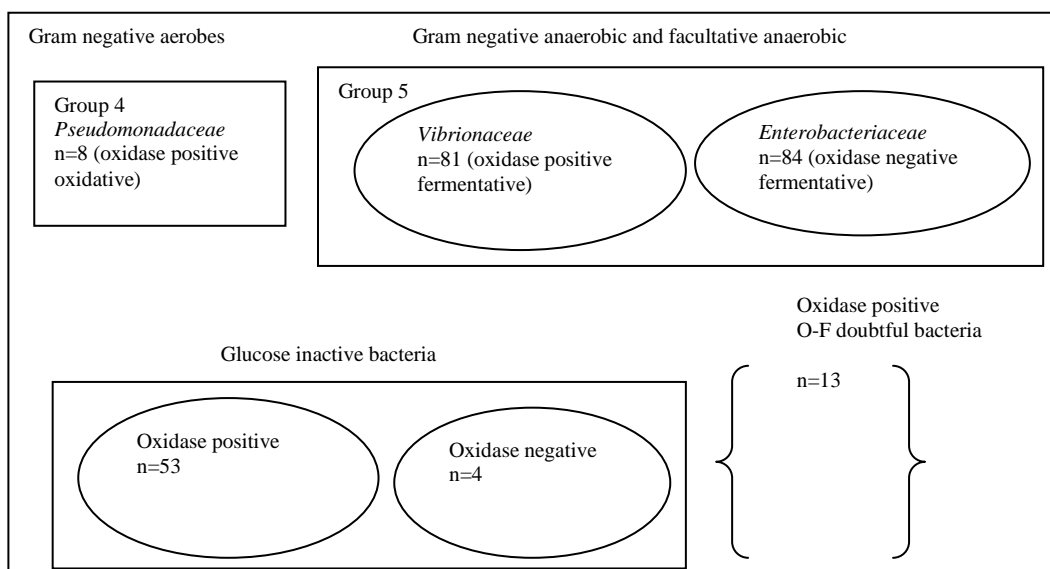
(55.5%) were suggested to belong to the genus *Vibrio*, thirteen (10.9%) to the genus *Shewanella*, and four (3.4%) to the genus *Aeromonas*. Thirty-one (26.1%) of the isolates were proposed to belong to the family of *Enterobacteriaceae*. Five of the isolates were closely aligned with *Pseudomonas* spp. (Table 4.3).

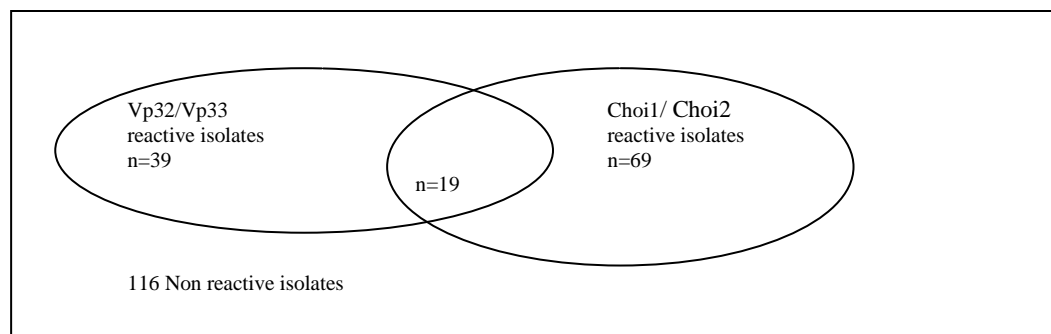4.4.2.3.2 Phylogenetic analysis

After all the partial 16S rRNA sequences were trimmed to yield a 591bp sequence for each isolate, two trees were constructed using the Neighbour joining method to group the unknown isolates. One tree consisted of 80 *Vibrionaceae* related isolates and the second tree consisted of the 30 isolates grouped as belonging to the *Enterobacteriaceae*. Four sequences were not included in the trees because they did not produce good sequences of the desired fragment size. Also five sequences belonging to *Pseudomonas* were excluded from the trees because they were very different from the sequences of the *Vibrionaceae* and *Enterobacteriaceae*.

The NJ method assigned the 80 *Vibrionaceae* to 14 clusters. Fifty-eight isolates grouped in cluster 1 with *V. alginolyticus, V. campbellii, V. natriegens, V. mytili, V. rotiferianus, V. diabolicus, V. parahaemolyticus* and *V. nereis* type strains. Four isolates grouped together in cluster 2, while one formed a separate group in cluster 3. Cluster 4 consisted of *V. fluvialis*, cluster 5 consisted of *V. harveyi* and cluster 6 consisted of *V. proteolyticus* type strains. None of the isolates clustered within these groups. Cluster 7 consisted of *V. albensis*, *V. cholera,* and *V. mimicus*. Again none of the isolates were found in these clusters. One isolate clustered within the *Listonella anquillarum* group (cluster 8).

Gram negative aerobes

Gram negative anaerobic and facultative anaerobic

Group 4
Pseudomonadaceae
n=8 (oxidase positive
oxidative)

Group 5

Vibrionaceae
n=81 (oxidase positive
fermentative)

Enterobacteriaceae
n=84 (oxidase negative
fermentative)

Oxidase positive
O-F doubtful bacteria

n=13

Glucose inactive bacteria

Oxidase positive
n=53

Oxidase negative
n=4

Figure 4.1 Potential groups where Gram negative bacteria isolated from TCBS could belong to. Four isolates were Gram positive

Vp32/Vp33
reactive isolates
n=39

Choi1/ Choi2
reactive isolates
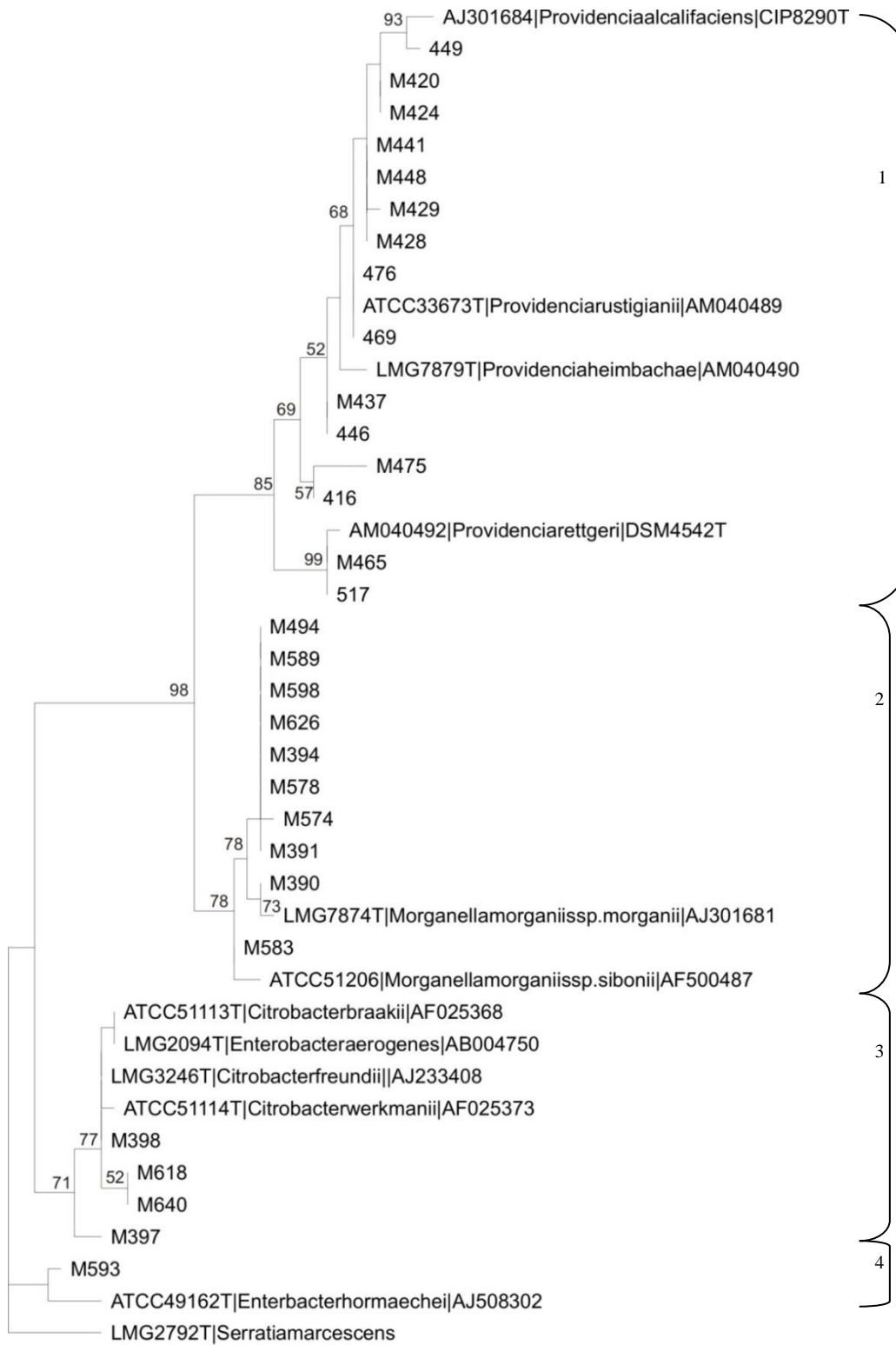n=69

n=19

116 Non reactive isolates

Figure 4.2 Grouping of isolates from TCBS that reacted with the *VP32/Vp33* and *Choi1/Choi2* primer pairs

Cluster 9 consisted of eight isolates belonging to the genus *Shewanella.* Three isolates clustered together with *Shewanella haliotis* in group 10. One of the isolates was found within cluster 11 together with *Shewanella algae*. *Shewanella putrefaciens* formed a separate cluster 12, but none of the isolates were found in this group. Cluster 13 was a distinct group consisting of the *Aeromonas veronii* and three isolates, cluster14 consisted of *Aeromonas hydrophyla* type strains with one isolate. Most of these groups were supported with high bootstrap values of between 55-100%. *Aeromonas punctata* was used as an outgroup (Figure 4.3). In the second tree the 30 *Enterobacteriaceae* isolates were divided into four groups; fifteen isolates clustered with *Providencia alcalifaciens*, *Providencia rustigianii*, *Providencia heimbachae* and *Providencia rettgeri* in group 1.Ten (10) isolates clustered together with *Morganella morganii* subspecies *morganii* and *Morganella morganii* subspecies *sibonii* in group 2. Four (4) isolates clustered together with *Citrobacter braakii*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Citrobacter werkmanii* in group 3. One isolate clustered with *Enterobacter hormaechei in* group 4. The four groups were supported by high bootsrap values of between 52 and 98. *Serratia marcescens* was used as an outgroup (Figure 4.4).

Table 4.3 Isolates that reacted with *Vp32/Vp33* and *Choi1/Choi2* primer pairs grouped according to their similarities to families and genera based on a BLASTn search.

Taxon identification based on BLASTn results	Number of isolates	Family	Genus
V. alginolyticus	31	*Vibrionaceae*	*Vibrio*
V. parahaemolyticus	22	*Vibrionaceae*	*Vibrio*
Vibrio spp	11	*Vibrionaceae*	*Vibrio*
V. harveyi	1	*Vibrionaceae*	*Vibrio*
Vibrio natriegens	1	*Vibrionaceae*	*Vibrio*
Shewanella alga	13	*Vibrionaceae*	*Shewanella*
Aeromonas hydrophyla	2	*Vibrionaceae*	*Aeromonas*
Aeromonas media.	1	*Vibrionaceae*	*Aeromonas*
Aeromonas molluscorum	1	*Vibrionaceae*	*Aeromonas*
Citrobacter freundii	3	*Enterobacteriaceae*	*Citrobacter*
Enterobacter aerogenes	2	*Enterobacteriaceae*	*Enterobacter*
Enterobacter spp	1	*Enterobacteriaceae*	*Enterobacter*
Enterobacteriaceae bacterium	1	*Enterobacteriaceae*	*Enterobacteriaceae*
Morganella morganii	10	*Enterobacteriaceae*	*Morganella*
Pectobacterium (Erwinia) carotovorum)	1	*Enterobacteriaceae*	*Erwinia*
Providencia alcalifaciens	3	*Enterobacteriaceae*	*Providencia*
Providencia heimbachae	2	*Enterobacteriaceae*	*Providencia*
Providencia rustigianii	8	*Enterobacteriaceae*	*Providencia*
Pseudomonas spp.	5	*Pseudomonadaceae*	*Pseudomonas*

Figure 4.3 Neighbour-joining tree of 80 isolates and 21 type strains belonging to the *Vibrionaceae* based on a 591 bp sequence of the 16S rRNA. Bootstrap values based on a 1000 repeats. *Aeromonas punctata* was used as an outgroup. Not all the clusters are indicated with brackets.

Figure 4.4 Neighbour-joining tree of 30 isolates and 21 type strains belonging to the family *Enterobacteriaceae* based on a591 bp sequence of the 16S rRNA. Bootstrap values based on a 1000 repeats. *Serratia marcescens* was used as an outgroup.

4.4.3 Confirmation of isolates identities

To confirm their identities the 63 isolates that clustered within the *V. parahaemolyticus/ V. alginolyticus group* were subjected to API 20E and a few other phenotypic tests (VP, ODC tests, L-Arabinose, cellobiose and sucrose fermentations as well as growth in 10% NaCl) to enable demarcation of the two species (Farmer et al., 2004; Oliver and Kaper, 1997).

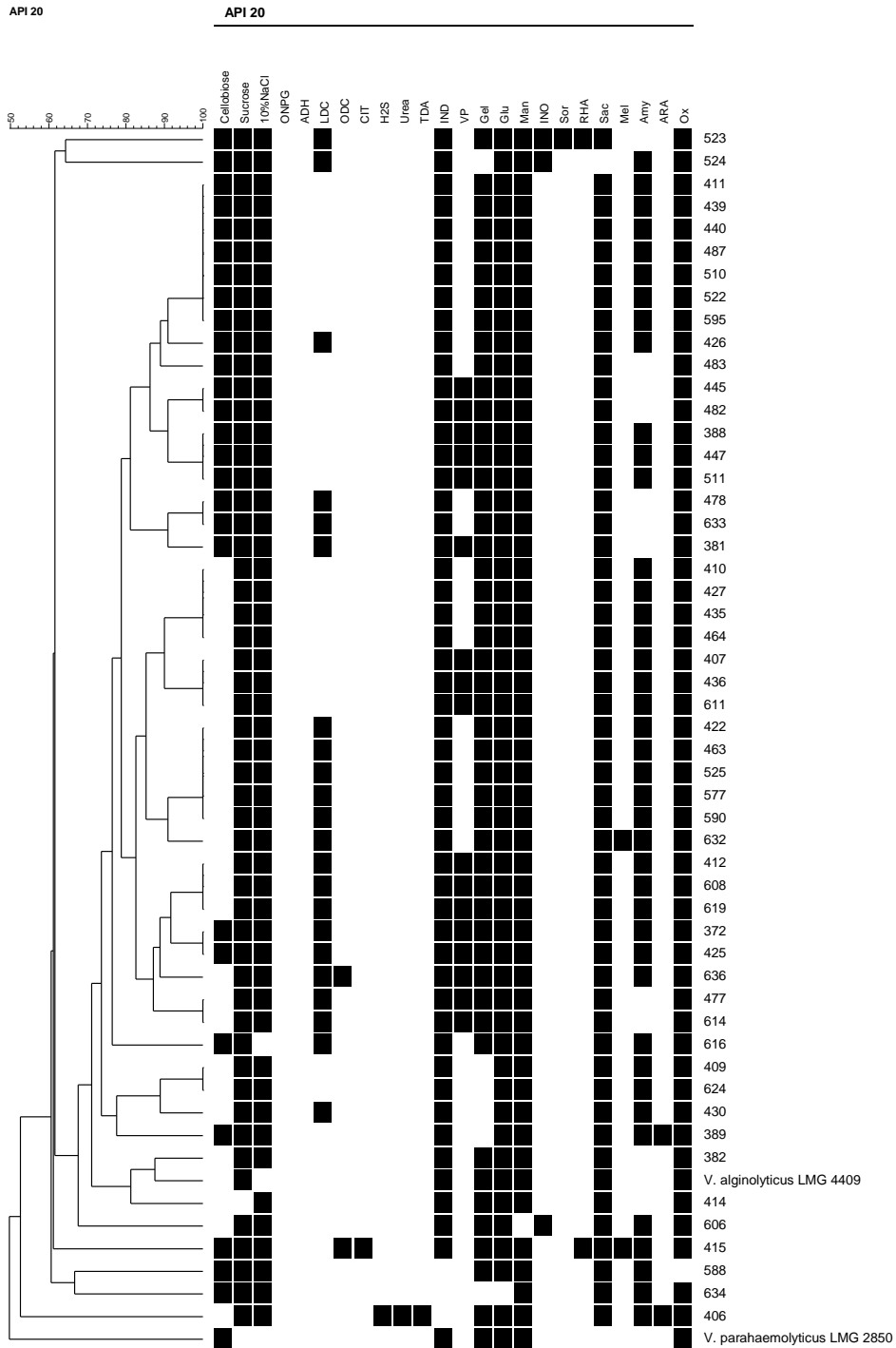4.4.3.1 API 20E and other biochemical tests

Based on the API lab software database 58 (92.1%) of the 63 isolates, for which it was impossible to distinguish between *V. Alginolyticus* or *V. parahaemolyticus* in the NJ method eighteen (18) of these isolates also aligned with *V. parahaemolyticus* in the BLASTn of their 16S rRNA sequences, were identified as *V. alginolyticus,* four (6.3%) (406, 407, 415, and 511) were identified as *A. hydrophyla* and one (634) was identified as *Pasteurella pneumotropica*. Twenty-six of these isolates were, however, only 49.5% similar to the standard *V. alginolyticus*. These isolates differed from the typical *V. alginolyticus* biochemical profile in tests such as cellobiose and amygladin fermentation, the VP and lysine decarboxylase (LDC) tests some of which are important in the differentiation of *V. parahaemolyticus* from *V. alginolyticus*. *V. alginolyticus* LMG4409 did not ferment cellobiose or amygladin and was VP and LDC tests negative.

4.4.3.2 Phenotypic comparison

The biochemical profiles of 52 selected isolates that clustered within the *V. Parahaemolyticus/ V. alginolyticus* group were imported into Bionumerics and a dendrogram was constructed using the Jaccard coefficient and unweighted pair group with arithmetic average (UPGMA) (Figure 4.5). *V. alginolyticus* LMG4409 and *V. parahaemolyticus* LMG2850 were used as controls. The examined isolates were analysed for 24 traits. All isolates clustered closer to *V. alginolyticus* LMG4409 and were clearly distinct from *V. parahaemolyticus* LMG2850. Isolate 406 differed substantially from the other *V. alginolyticus* strains as it fermented arabinose, produced urease and H$_2$S. It was also TDA (tryptophan deaminase) positive and the isolate did not produce indole from tryptophan (Figure 4.5)

4.4.3.3 *V. alginolyticus* specific collagenase gene PCR.

According to the BLASTn results 22 of the isolates that amplified the *pR72H* fragment, were potential *V. parahaemolyticus* isolates. These isolates were screened for the *V. alginolyticus* specific collagenase gene. Only three (13.6 %) of the isolates did not react with *V. alginolyticus* specific collagenase gene primers. Nineteen of these isolates produced the 737 bp fragment of *V. alginolyticus* collagenase gene. *V. alginolyticus* LMG4409 gave the same reaction. The type strains of *V. parahaemolyticus* LMG2850, *V. vulnificus* LMG 13545 and an environmental strain of *V. cholerae* C453 did not react (results not shown).

Figure 4.5 Dendrogram showing the levels of phenotypic similarity amongst the 52 selected *Vibrio* species isolates where a clear differentiation between *V. parahaemolyticus* and *V. alginolyticus* could not be made based on the 16S rRNA sequence data.

4.4.4 Final identities of isolates that were selected from TCBS.

Of all the isolates investigated 4 were Gram positive and 243 were Gram negative. The initial grouping of the sucrose negative and sucrose fermenting isolates according to the bacteria families they belonged to are shown in Table 4.4. Out of the 243 isolates fifty-two were confirmed to be *V. alginolyticus* according to the combined results. The other *Vibrionaceae* could be *V. campbellii, V. natriegens*, *V. mytili*, *V. rotiferianus*, *V. diabolicus*, *V. nereis*, or *Listonella anquillarum*. The *Enterobacteriaceae* belonged mainly to *Providencia*, *Morganella* and *Citrobacter* genera.

The majority of the isolates belonged to the family *Vibrionaceae* (*Vibrios, Shewanella)* and *Aeromonadaceae* followed by the family *Enterobacteriaceae*. Sucrose negative isolates that showed nonspecific amplification of the *pR72H* fragment of *V. parahaemolyticus* and the cytolysin–haemolysin gene of *V. Vulnificus* were *Shewanella*, *Morganella*, *Aeromonas*, *Providencia* spp. and *Pseudomonadaceae*.

Table 4.4 Grouping of 247 bacteria isolated from TCBS grouped based on the sucrose fermentation

Gram	Sucrose	Heading	Isolates	Number	Family
negative	positive	Oxidase+ve and O	459, 460, 484	3	*Pseudomonadaceae*
		Oxidase +ve and OF/F	341, 372, 374, 377, 381, 382,383, 386, 388, 399, 406, 407, 409, 411, 415, 419, 421, 423, 434, 438, 440, 461, 462, 464, 481, 488, 490, 499, 502, 509, 510, 521, 522, 523, 524, 525, 526, 528, 530, 531, 532, 533, 534, 535, 536, 541, 542, 544, 545, 546, 547, 548, 590, 594, 595, 606, 608, 611, 614, 619, 632, 633, 634, 635, 638, 645, 648, 649	68	*Vibrionaceae*
		Oxidase+ve and Glu⁻ve	343, 379, 401, 402, 404, 412, 432, 435, 436, 454, 455, 463, 482, 483, 486, 495, 504, 511, 512, 516, 518, 543, 553, 570, 572, 586, 588, 597, 612, 616, 623, 624	32	*Pseudomonadaceae* *Vibrionaceae*

Table 4.4 continued

Gram	Sucrose	Heading	Isolates	Number	Family
negative	positive	Oxidase-ve and OF/F	389, 400, 403, 405, 408, 424, 441, 445, 449, 529, 538, 593	12	*Enterobacteriaceae*
		Oxidase-ve and Glu-ve	452, 644,	2	*Enterobacteriaceae*
		Oxidase+ve and doubtful	410, 422, 425, 426, 427, 430, 439, 447, 478, 487, 577, 636,	12	*Vibrionaceae*
	Sucrose negative	Oxidase+ve and O	568, 582, 617, 628, 629	5	*Pseudomonadaceae*
		Oxidase +ve and OF/F	385, 431, 443, 451, 468, 472, 479, 480, 496, 500, 520,	11	*Vibrionaceae*
		Oxidase+ve and Glu⁻ve	467, 473, 501, 514, 537, 550, 551, 569, 571, 575, 576, 596, 599, 600, 601, 602, 613, 615, 622, 641, 642	21	*Pseudomonadaceae* *Vibrionaceae*

Table 4.4 continued

Gram	Sucrose	Heading	Isolates	Number	Family
negative	negative	Oxidase-ve and OF/F	384, 387, 390, 391, 392, 394, 396, 397, 398, 417, 418, 433, 437, 446, 448, 465, 466, 469, 475, 476, 477, 492, 493, 494, 497, 498, 503, 505, 506, 507, 515, 517, 519, 527, 539, 540, 549, 574, 578, 579, 580, 581, 583, 584, 585, 587, 589, 591, 592, 598, 603, 604, 605, 607, 609, 610, 618, 620, 621, 637, 639, 643, ,640, 626, 630, 631, 646, 647	68	*Enterobacteriaceae*
		Oxidase-ve and Glu-ve	416, 420, 428, 429, 453, 456, 457, 513	8	*Enterobacteriaceae*
		Oxidase+ve and doubtful	414	1	*Vibrionaceae*

Table 4.4 continued

Gram positive	Sucrose positive	Heading	Isolates	Number	Family
		Oxidase+ve and O	444	1	*Micrococci*?
		Oxidase-ve and NR	450	1	*Micrococci*?
	Sucrose negative	Oxidase+ve and O	458	1	*Micrococci*?
		Oxidase – ve and OF	380	1	*Streptococcus*?

Abbreviations: Oxidase+ve and O= Oxidase positive and oxidative, Oxidase +ve and OF/F = Oxidase positive and fermentative, Oxidase+ve and Glu⁻ve = oxidase positive and glucose inactive, Oxidase-ve and OF/F = Oxidase negative and fermentative, Oxidase-ve and Glu-ve= oxidase negative and glucose inactive, Oxidase+ve and doubtful= oxidase positive and glucose fermentation doubtful.

4.5 DISCUSSION

From a consumer health perspective it was important to establish the identities of the bacterial species associated with processed hake, pilchard and horse mackerel. *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. The diseases associated with human pathogenic *Vibrio* species; (cholera, gastroenteritis, septicaemia and wound infection) cause huge economic losses and sometimes are fatal (Thompson et al., 2004b). The fatality rate in *V. vulnificus* infections is 40-50% (Hsueh et al., 2004). In addition the presence of human pathogenic *Vibrio* species in these fish species creates an impediment in international trade. According to the current trade agreement hake would be rejected in case *V. cholerae* was detected in the fish by the recipient countries especially those of the EU.

TCBS is one of the recommended media used for the selective isolation of *Vibrio* species (Ottaviani et al., 2003). The selectivity of this medium, may, however, vary (Sakazaki and Ballows, 1991). *Vibrios* form characteristic colonies (yellow or green) based on the ability of the isolate to ferment sucrose. In certain instances TCBS medium may be too inhibitory and suppresses the growth of some species of *Vibrio*. Bacteria other than *Vibrios* such as *Enterobacteriaceae*, *Proteus*, *Aeromonas* and *Staphylococci* may also grow (Joseph et al., 1982). During this study both sucrose and sucrose non fermenting bacteria were identified because both groups are found as human pathogens often transmitted to humans via consumption of fish or through sea water (Drake et al., 2007). In addition the three human pathogenic *Vibrio* species do not have specific sucrose fermenting characteristics (Arias et al., 1998; Joseph et al., 1982; Nagao et al., 2006; Oliver et al., 1992;).

Of the bacteria isolated from TCBS 243 isolates belonged to the class γ-Proteobacteria, (Holt et al., 1994), and only four isolates were Gram positive confirming that TCBS is not 100% selective for Gram negative bacteria as was also found by others (Gomez-Gil and Roque, 2006). Gram positive bacteria form an important part of the bacterial

population on fish. It has been reported that isolates from the genus *Micrococcus* were part of the predominant bacteria on fish from warm waters (Genarri et al., 1999). *Moraxella, Pseudomonas, Corynebacterium* and *Micrococcus* were isolated from hake fish of the Atlantic waters (Vennemann et al., 1994). Out of 243 isolates 133 were sucrose fermenters while 110 were sucrose non fermenters. However pathogenic *Vibrio* species belong to both groups and variability within a species with regard to sucrose fermentation is common; hence the sucrose fermentation test is not an important trait in the taxonomy of *Vibrio* species. In addition this test was unable to differentiate *Vibrio* species from *Enterobacteriaceae* and *Pseudomonadaceae*. Although some sucrose negative colonies were isolated no typical *V. parahaemolyticus* colonies were detected on TCBS.

After the Gram stain differentiation, the first step to group and identify the strains isolated from TCBS was to carry out the oxidase and O-F tests. A substantial number of the bacteria isolated during this study, 53 (21.8%) were oxidase positive and glucose inactive, while 4.1% were oxidase positive, oxidative and possibly belonging to the family *Pseudomonadaceae.* The 16S rRNA gene sequence alignment indicated that some of these isolates did belong to the genera *Vibrio* and *Shewanella* while others belonged to the *Enterobacteriaceae*. The inability of these isolates to ferment glucose confirms the unusual nature of the bacteria isolated from marine waters. This study has isolated four bacterial families using TCBS; *Vibrionaceae*; (*Vibrios* and *Shewanella)*, *Aeromonadaceae, Enterobacteriaceae* (*Morganella*, *Providencia*, *Enterobacter* and *Citrobacter*) and *Pseudomonadaceae*.

Confirmation of the *Vibrio spp*. identity is normally carried out by biochemical tests and by serological methods. Cultural methods, however, have disadvantages such as delays in obtaining final results, and that atypical isolates are difficult to identify due to variations in important phenotypic characteristics. This approach has in many laboratories been superseded by molecular methods that identify bacteria at the gene level or by protein profiling.

In this study it was decided to screen all isolates directly using the species specific PCRs for the three selected human pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) regardless of the sucrose fermentation properties of the isolates. The main reason for this decision was that strains with atypical sucrose fermentation patterns have been reported for all three of these species. A few strains of *V. parahaemolyticus* (Joseph et al., 1982) and *V. vulnificus* (Arias et al., 1998; Nagao et al., 2006; Oliver et al., 1992) have been reported to ferment sucrose. On the other hand sucrose negative *V. cholerae* O139 strains have also been documented (Ansaruzzaman et al., 1995).

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. This study has shown that the *pR72H* fragment and the *V. vulnificus* specific cytolysin-haemolysin genes are widely distributed amongst *Vibrionaceae* and *Enterobacteriaceae*. While the Phosphatidyl serine synthetase gene (Lee et al., 1995) is present in *V. alginolyticus* and few *Shewanella* species, parts of the cytolysin-haemolysin sequence of *V. vulnificus* (Lee and Choi, 1995) are widely distributed among other species of *Vibrionaceae* including *Shewanella* and *Aeromonas* as well as amongst the *Pseudomonadaceae*. A number of *Enterobacteriaceae* species including *Providencia*, *Morganella* and *Citrobacter* also carry this gene. *V. alginolyticus* was the most predominant species that carries sequences belonging to both of these genes. The reaction of *V. alginolyticus* strains to *V. parahaemolyticus* specific genes has confirmed previous findings where this was observed (Gonzales–Escalona et al., 2006; Robert-Pillot et al., 2002; Xie et al., 2005). It was, however, the first time that the *V. vulnificus* specific cytolysin-haemolysin based sequences were being detected in other bacterial species.

The problem encountered in detecting genes in different species using species specific primers could possibly be attributed to a high frequency of gene mobility in marine environments. Environmental strains might also be more prone to genetic changes due to natural selection processes in unfavourable environments. Tests used to accurately

identify clinical strains might therefore not be appropriate for bacteria isolated from the environment. It was therefore necessary to use supplementary molecular methods (detection *V. alginolyticus* specific collagenase gene), and phenotypic data so as to validate the results obtained with the initial phenotypic tests (API 20E), and from the contradictory genotypic results (detection of the *pR72H* fragment and the cytolysin-haemolysin gene for *V. parahaemolyticus* and *V. vulnificus* respectively).

As a follow-up the partial 16S rRNA sequences were determined for these strains. The 16S rRNA sequences, however, did not offer final identifications due to a high level of interspecies 16S rRNA nucleotide sequence homology displayed within the genus *Vibrio* (Thompson et al., 2005). More than 19% of the *V. alginolyticus* isolates tested had between 97 and 100% 16S rDNA sequence homology with *V. parahaemolyticus*. The 16S rRNA sequences did help to differentiate *Vibrio* species from other *Vibrionaceae* such as *Aeromonas* and *Shewanella*. The sequences were also used to differentiate the *V. parahaemolyticus*/ *V. alginolyticus* group from the *V. cholerae, V. mimicus* group, but it was unable to differentiate *V. parahaemolyticus* from *V. alginolyticus*.

Identification of *Vibrio* species especially differentiation between *V. alginolyticus* and *Vibrio parahaemolyticus* by phenotypic tests is difficult because the two species have similar characteristics. The most common test used is their ability to ferment sucrose, but final identification is based on the VP, urease, cellobiose, dulcitol, sucrose, ONPG, Arabinose test and growth in 10-12% NaCl (Farmer et al., 2004; Oliver and Kaper, 1997;). Even these tests do not give clear cut differences between the two species since some strains still behave differently.

To resolve the contradiction of the sixty-three isolates that clustered within the *V. alginolyticus V. parahaemolyticus* group a polyphasic approach was applied to obtain the final identities of isolates to species level. The results from the phylogenetic trees were compared with those from the API lab software database and by determining their phenotypic similarities. The *V. alginolyticus* collagenase specific PCR was also included. API 20E identified 55 isolates from the *V. alginolyticus- V. parahaemolyticus* group in

the phylogenetic tree as *V. alginolyticus*. Only one isolate was misidentified as *Pasteurella pneumotropica* while the second was misidentified as *A. hydrophyla*. The collagenase specific gene gave the same results, but three isolates were negative for this gene. This is in line with the work of Cai et al. (2009) who demonstrated that this gene is only present in fish pathogenic *V. alginolyticus* strains. During this study specific phenotypic tests assisted the most in differentiating between *V. parahaemolyticus* and *V. alginolyticus*. This finding is in line with the study of West et al. (1986) who used biochemical characters and were able to clearly differentiate the two species using the same methods as we used. Some *V. alginolyticus* strains isolated during this study did not ferment glucose. This finding is in contradiction with the findings of West et al. (1986) where all species of *Vibrios* were found to ferment glucose. This situation stresses the unusual character of the isolates from marine fish processing plants.

V. alginolyticus strains isolated from processed marine fish ready for distribution were found to carry sequences belonging to *V. parahaemolyticus* (*pR72H* fragment and the *trh*) an important pathogen transmitted through shellfish and responsible for gastroenteritis in humans. Robert-Pillot et al. (2002) stressed that the pR72H gene fragment is confined to *V. parahaemolyticus,* and its detection is strongly associated with this species. It is likely that these isolates acquired foreign genetic material from the marine environment due to its dynamic nature. The evolution of pathogens in the environment is believed to take place as a result of horizontal gene transfer between pathogenic and non pathogenic strains (Vezzulli et al., 2008). The fish isolated *V. alginolyticus* strains could therefore have the potential to cause gastroenteritis normally associated with *V. parahaemolyticus*. The high phenotypic and genetic similarity of *V. alginolyticus* isolates to *V. parahaemolyticus* could also indicate the ability of the virulent strains of *V. parahaemolyticus* to thrive in processed marine fish under similar conditions. This situation is a cause of concern and could pose a health threat to consumers.

4.6 CONCLUSION

The taxonomy of *Vibrios* and related genera is a complex and controversial subject that is subjected to constant changes depending on the current findings and improvements in taxonomic methods (Ruimy et al., 1994). 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 the three examined fish species are safe and do not pose a health hazard to consumers. This study confirms that enrichment in APW and GSTB and plating on TCBS can still be used in isolating *Vibrio* species from marine samples, but the selectivity of TCBS needs to be improved to minimise growth of other bacteria that are not *Vibrios* such as *Pseudomonas, Aeromonas*, *Shewanella* and *Enterobacteriaceae*. The *V. parahaemolyticus* and *V. vulnificus* primers are not specific and therefore were not useful in differentiating between *Vibrio* species. Both the *pR72H* fragment and the cytolysin haemolysin gene primers also showed non specific binding with some of the *Enterobacteriaceae* and *Pseudomonadaceae* isolates. Neither the classical method nor the detection of species specific genes alone is 100% reliable in identifying *V. alginolyticus* and *V. parahaemolyticus* to species level. However the PCR targeting the *ompW* of *V. cholerae* continues to be a valuable tool to differentiate between sucrose fermenting strains of *V. cholerae* and *V. alginolyticus*. The use of the NJ method of the 16S rDNA in combination with biochemical profiles from API20E facilitates accurate identification of *Vibrio* species.

4.7 RECOMMENDATIONS

For identification of *Vibrio,* particularly when differentiating *V. alginolyticus* and *V. parahaemolyticus*, a polyphasic approach is always recommended as a baseline using phenotypic tests such as the API 20E combined with PCR for species specific genes such as the collagenase gene of *V. a*l*ginolyticus*, so as to avoid ambiguity in the final results. *Vibrio alginolyticus* is an important fish pathogen and some of its strains may carry *V. parahaemolyticus* virulence associated sequences (Gonzales–Escalona et al., 2006; Xie et al., 2005). Some strains of *V. alginolyticus* are important fish pathogens in aquaculture (Balebona et al., 1998; Liu et al., 2004) while others are used as probiotics in the same systems (Austin et al., 1995; Balcázar et al., 2006; Verschuere et al., 2000). Accurate identification of this species is therefore very important. Further studies need to be carried out to establish the role of *V. alginolyticus* in marine fish processing factories. Pathogenicity studies could also be carried out in animal models to determine its safety implications on consumers.

4.8 ACKNOWLEDGEMENTS

4.9 REFERENCES

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