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Review

The role, isolation and identification of *Vibrio* species on the quality and safety of seafood

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Seafoods in their natural environments are associated with a variety of microorganisms. Fish shelf life reduction results from microbial metabolism, mainly by Gram negative bacteria that produce chemical compounds responsible for bad odour, texture and taste. Shelflife is estimated by performing total viable bacterial counts at ambient and refrigeration temperatures. The type and number of bacteria present on seafood depends on the microbial composition of the surrounding waters, on the intrinsic factors, extrinsic factors, processing, and implicit factors and on the microbial interactions within the fish itself. Although, sea food safety assessment is preferably determined by detecting indicator organisms; such as Enterobacteriaceae and coliforms, none of these groups fulfil all requirements that guarantee food safety necessitating direct detecting of relevant pathogens. *Vibrio* species are part of the bacteria genera associated with seafoods borne diseases. Prompt and accurate detection and identification methods of pathogens are imperative to determine the product compliance with seafood microbiological criteria. Although cultural methods have long been used in detecting human pathogens including *Vibrio* species in fish, these methods are time consuming and sometimes inaccurate. Also some pathogens have the propensity to change into the Viable but non culturable (VBNC) state in unfavourable environments. The use of molecular methods is hampered by drawbacks, such as inter species 16S rRNA sequence similarity and that some strains carry multiple copies of the 16S rRNA gene. A combination of classical, numerical taxonomy and Multi locus sequence analysis (MLSA) methods are promising to give absolute resolution between closely related *Vibrio* species.

Key words: *Vibrios* spp., seafood, spoilage, pathogens, detection, identification.

INTRODUCTION

Seafood spoilage is of biochemical and /or microbial origin and results in limited shelf life and the eventual sensory rejection of the food (Gennari et al., 1999; Gram, 1992; Gram and Dalgaard, 2002; Huis in't Veld, 1996). Bacterial counts performed at different incubation temperatures and detection of indicator organisms usually indicate the degree of contamination and are used to predict the sanitary conditions under which the food is produced and to estimate the microbiological quality of a food item (ICMSF, 1978). The specific bacteria that carry out metabolism in the fish muscle after death depend on the type of fish and its chemical composition, the feeding habit, the area where the fish is harvested and the type of fishing gear used during harvesting (Françoise, 2010; Huis in't Veld, 1996). The storage conditions dictate the microbial successions and the final predominant groups (Gram and Huss, 1996; Huis in't Veld, 1996). *Vibrio, Shewanella, Aeromonas,* Enterobacteriaceae and *Photobacterium* are psychrotrophic and play important roles in the quality and shelf life of marine fish during storage (Koutsomanis and Nychas, 1999). *Aeromonas, Bacillus, Campylobacters, Clostridium, Mycobacterium, Legionella, Edwardsiella, Plesiomonas, Salmonella,*

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Yersinia and *Vibrios* are common seafood borne human pathogens. They cause diseases including gastroenteritis, septicaemia and wound infections that are sometimes fatal, botulism and scombrotoxicosis (Novotny et al., 2004). *Vibrio* species bacteria are ubiquitous in aquatic environments including fresh, coastal and marine habitats. They are also found as commensals on the surfaces and in the digestive tracts of fish and in zooplanktons (Drake et al., 2007; Montanari et al., 1999). They are transmitted to humans via raw or improperly cooked fish or contaminated water. The resultant diseases are either toxigenic or infectious. However, scombrotoxicosis caused by some species of *Vibrios* is a Type I hypersensitivity reaction mediated by histamine the metabolic end product of the amino acid histidine found abundant in scombroidea fish species.

Vibrio species testing is part of the microbiological criteria for marine fish in international trade. Apart from their ubiquity in aquatic environments some *Vibrio* species are emerging pathogens that cause up to more than 50% deaths of all clinical cases (Hsueh et al., 2004; Novotny et al., 2004). *Vibrio cholera* and *Vibrio parahaemolyticus* are sensitive to moderate heat treatment 45 to 60℃ while *Vibrio vulnificus* responds well to acid treatment.

Laboratory efforts of detecting *Vibrios* have for many decades depended on cultural means followed by identification by biochemical tests. This approach was suitable as most *Vibrio* species are non fastidious, although a few have specific nutritional requirements (Thompson et al., 2004) while others may be undetectable during dormant states (Wong and Wang, 2004). The initial step in *Vibrio* isolation is resuscitation in alkaline peptone water (APW) pH 8.6, followed by plating on thiosulphate citrate bile salts sucrose (TCBS) agar as the differential selective media on which sucrose fermenting and non sucrose fermenting species are differentiated. Other media such as brain heart infision (BHI) broth and cellobiose polymyxin B colistin (CPC) agar for *V. vulnificus* have been designed. Problems encountered during *Vibrio* isolation have led to refinement of cultural methods and to the design of molecular based methods including protein profiling; the detection of species specific genes and in some instances a polyphasic approach.

The present document is a review of the literature about microbiological quality issues encountered in fish factories with specific emphasis on three most important human pathogenic *Vibrio* species; their biology and survival patterns, the available methods of detection and identification are highlighted. The uniqueness of this paper is that it presents the current state of the art, summarises the challenges met and it uses information from various research groups to bridge the gaps, shortfalls and uncertainties commonly experienced in the field. The first part discusses the consequence of metabolic activities of these microorganisms in fish and the interpretation of microbial load to public health. The second part explores the importance of human pathogens transmitted via the consumption of contaminated fish. Their ecology, clinical manifestations in humans, methods of isolation and identification are discussed. Problems encountered and progress made with the use of different methods has also been reviewed.

SPOILAGE OF SEAFOOD

Fish spoilage occurs as a result of autolysis and lipolysis due to the activity of endogenous enzymes or contamination by metabolically active microorganisms (Chang et al., 1998; Ordóñez et al., 2000; Chytiri et al., 2004). Bacteria are pivotal in the process of seafood spoilage by either initiating or accelerating the spoilage process (Gram, 1992; Gennari et al., 1999; Tryfinopoulon et al., 2002).

The number of total viable bacteria is a measure of the general microbiological quality of the food. High mesophilic counts indicate poor sanitary practise and temperature abuse during processing hence signaling a health hazard. Mesophilic counts however depend on the type of food and number present during harvesting; it may also indicate that a slow spoilage process is taking place. The pshychrotrophic count may indicate the shelf life under refrigeration storage (ICMSF, 1978; Mol et al., 2007). Psychrotrophic bacteria belonging to the class γ-Proteobacteria group are well documented as spoilage organisms in fresh and preserved fish (Chytiri et al., 2004; Françoise, 2010; Gennari et al., 1999; Gram and Huss, 1996; González et al., 1999; Himelbloom et al., 1991; Koutsoumanis and Nychas, 1999; Ordóñez et al., 2000; Tryfinopoulou et al., 2002). The total viable aerobic plate count (TVC) typically ranges between 10^2-10^6 cfu/g/cm^2 in fresh fish (Chang et al., 1998; Giménez et al., 2002; Mahmoud et al., 2004; Popovic et al., 2010). Fish spoilage is usually associated with total counts of 10^7 to 10^8 of the specific spoilage organisms (Gram and Huss, 1996; Ordóñez et al., 2000).

Fresh fish contain 10^4 to 10^6 cfu/g on the skin, 10^4 to 10^7 in the gills (Gennari et al., 1999), and 10^3 to 10^5 in the intestines (Nickelson and Finne, 1992). Studies carried out on fresh fish and ice-stored fish from temperate regions show that the predominant microflora include *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Brochotrix thermosphacta*, *Pseudomonas* species, *Aeromonas* species and lactic acid bacteria (LAB) (Chytiri et al., 2004; Françoise, 2010; Tryfinopoulou et al., 2002). Similar studies on initial microflora on sardines from the Adriatic Sea found *Pseudomonas fluorescence*, *Pseudomonas putida*, *Shewanella putrefaciens, Achromobacter, Acinetobacter, Psychrobacter* and *Flavobacterium* (Gennari et al., 1999). Fish from warm

waters mainly carries Gram-positive bacteria such as coryneforms, micrococci and Enterobacteriaceae as the predominant microflora (Gennari et al., 1999). *Shewanella* and *Pseudomonas* are the predominant microorganisms in seafood stored on ice under aerobic conditions regardless of its origin (Gram and Huss, 1996; Ordóňez et al., 2000). Kyrana and Lougovois (2002) found *Vibrio* species and Enterobacteriacea as the major spoilage organism on sea bass stored at ambient temperatures. However, only 0.01 to 2% of the the total microflora are culturable, hence total viable counts do not represent absolute numbers and identities of the bacteria present (Françoise, 2010).

Factors that influence these microbial identities including contamination from the environment are the intrinsic factors (water activity, pH, nutritional composition of the fish, and redox potential), the extrinsic factors (temperature, surrounding environment in packages), the processing factors (lightly versus heavily preserved fish, slicing and grinding, the methods of preservation) and implicit factors (conditions of storage, transport and distribution, the biochemical reactions within the residual groups, and their interactions; synergism, antagonism) (Gram and Huss, 1996; Gram and Dalgaard, 2002; Huis in't Veld, 1996; Tryfinopoulou et al., 2002). Seafood spoilage may be minimised through adequate temperature control. Lactic acid bacteria may be used to inhibit spoilage bacteria during preservation and can significantly prevent spoilage by putrefaction (Sudalayandi and Manja, 2011). However, this approach still needs to be verified by further studies due to possible associated side effects including the selective growth promoting effect on psychrotrophic pathogens including *Listeria monocytogenes* and *Clostridium botulinum* type E (Françoise, 2010).

MICROBIAL SAFETY OF SEAFOOD

Apart from spoilage the safety of seafood also has to be controlled in terms of the presence of possible food–borne pathogens such as the human pathogenic *Vibrio* species, *C. botulinum*, *Aeromonas hydrophylla*, *B. cereus*, *Salmonella* spp., *Y. enterocolitica*, *L. monocytogenes* (Françoise, 2010). In 1978, 10.5% of all disease outbreaks and 3.6% of all case of seafood diseases in the United States of America were linked to the consumption of both shellfish and fin fish (Wekell et al., 1994).

Enterobacteriaceae counts are used as a measure of the degree of sanitation in food. High counts are a result of unsanitary handling or temperature abuse. The numbers do not always correlate with the extent of contamination from the original source, due to their ability to grow at varying rates in different food commodities (ICMSF, 1978). The coliform group is not well defined and results obtained can vary depending on the specimen, growth medium used, incubation temperature and methods used to read results. Human pathogens of exogenous origin in seafood include *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Campylobacter* spp*., Staphylococcus aureus* and *Bacillus cereus* introduced through poor personal hygiene or cross contamination with other contaminated foods (Popovic et al., 2010; Wekell et al., 1994)

Biogenic amines

Biogenic amines occur in a wide range of fresh and processed foods including cheese, sauerkraut, wine, liver, leafy vegetables, fruits, milk, chocolates, and meats (Chong et al., 2011; Karovičová and Kohanjdová, 2005; Santos, 1996). Some biogenic amines are natural constituents of the foods; others are a result of endogenous enzymes and or microbial metabolism. The nature and amount of the biogenic amine formed in a particular food depends of the specific food chemical composition and the types of microorganisms present (Santos, 1996). The proteinaceous nature of fish favours formation of biogenic amines some of which may cause intoxication in humans (Karovičová and Kohanjdová, 2005; Santos, 1996). Biogenic amines have been detected in seafoods including fresh fish, fermented fish and fish pastes. The most common of these amines include agmantine, cadaverine, putrecine, phenylethylamine, histamine, serotonin, spermine, spermidine and tryptamine, tyramine (Naila et al., 2011; Rabie et al., 2011).

In living tissues under normal conditions biogenic amine have a diverse role of synthetic and metabolic functions; as a source of nitrogen, precursors for the synthesis of hormones, alkaloids, nucleic acids and proteins, amines and as a source of aroma in foods. In humans they are also important as chemical messengers, and in regulating the blood pressure. Putrecine, cadaverine and spermine are important as free radical scavengers and antioxidants. However, some biogenic amines (agmatine, spermine and spermidine) can react with nitrites to form pro-carcinogenic nitrosamines (Santos, 1996) while others (histamine) in high concentrations trigger allergic reactions known as scombrotoxicosis in humans (Davis and Henry, 2007) associated with scombroid fish species (tuna and horse mackerel, and sardines) (Karovičová and Kohanjdová, 2003; Santos, 1996) and with other fish species such as salmon (Auerswald et al., 2006) as well as other food types such as cheese (Karovičová and Kohanjdová, 2003; Santos, 1996). Histamine is the most toxic biogenic amine known. Putrecine, cadaverine and agmatine suppress oxidation of histamine enhancing histamine toxicity (Chong et al., 2011; Santos, 1993). Biogenic

amines are formed mostly through decarboxylation of amino acids, through amination and trans-amination of ketones and aldehydes mainly by bacteria decarboxylases than by food endogenous enzymes (Karovičová and Kohanjdová, 2003; Santos, 1996). Bacteria of the genera Enterobacteriaceae, *Lactobacillus, Bacillus, Citrobacter, Proteus, Pseudomonas, Salmonella, Shigella, Klebsiella, Escherichia, Pediococcus, Streptococcus, Staphylococcus, Vibrio species*, *Raoultella planticolla* and some *Clostridia* are known to produce biogenic amines from their precursor molecules (Kanki et al., 2007; Santos, 1996). *Proteus morganii*, *Klebsiella pneumoniae, Klebsiella oxytoxa Hafnia alvei, Staphylococcus hominis* and *Enterococcus hirae* produce histamine at a fast rate and are important in the microbiological quality of fish, while *Photobacterium phosphoreum* and *Photobacterium damsela* are psychrotolerant and mesophiles respectively that produce biogenic amines in scombroid fish species at low temperatures and at ambient temperature (Economou et al., 2007; Kanki et al., 2007; Santos, 1996). Higher levels of histamine are found in improperly stored scombroid fish species due to high levels of histidine in these fish muscles (Auerswald et al., 2006; Karovičová and Kohanjdová, 2005). Apart from histamine, putrecine, cadaverine, tyramine, spermine and spermidine have also been detected in mackerel, herring, tuna, and sardines (Santos, 1996), tryptamine, 2-phenylethylamine, agmatine and serotonin in sea bass (Öxogul et al., 2006).

Its accumulation in fish muscle is commonly caused by temperature abuse during fish harvesting, processing, transport and storage, for example, when the fish is held at temperatures above 7℃ for several hours (Auersw ald et al., 2006; CDC, 2007; Economou et al., 2007) the rate of formation also depends on bacterial count (Takahashi et al., 2003). The amine is resistant to freezing and cooking (Chong, 2011). Formation of biogenic amines may be controlled by controling bacterial growth and by a number of food processing techniques including modified atmosphere packaging, food irradiation, high temperature treatment, addition of starter cultures that break down histamine and by hydrostatic presure (Chog et al., 2011; Naila et al., 2010). The levels of allowable histamine concentrations in food range from 50 to 200 ppm in Australia and USA respectively (Auerswald et al., 2006).

Vibrio species as food-borne pathogens

Vibrios are responsible for a number of clinical conditions such as cholera, gastroenteritis, septicaemia and wound infections (Jay et al., 2005; Oliver and Kaper, 1997; Thompson et al., 2004).Twelve *Vibrio* species have been documented as potential food-borne disease agents in humans: *V. cholerae*, *V. parahaemolyticus, V. vulnificus, Vibrio alginolyticus, Vibrio funissii, Vibrio fluvialis, Vibrio damsela, Vibrio mimicus, Vibrio hollisae, Vibrio cincinatiencis, Vibrio harveyi* and *Vibrio metchnikovii* (Adams and Moss, 2008; ICMSF, 1996; Thompson and Swings, 2006). A few species are pathogens of fish, while some other species are involved in coral bleaching (Thompson et al., 2004).

Vibrio species are transmitted to humans mostly via sewage contaminated water or seafood (finfish, molluscs and crustaceans) when consumed raw or partially cooked (De Paola et al., 2000; ICMSF, 1996; Oliver and Kaper, 1997). Though *Vibrio* species have been isolated from marine environments, poor processing practises are regarded as the major cause of the food contamination (Kaysner et al., 1992). The bacteria may persist in the food depending on storage temperatures, pH and the product water activity (ICMSF, 1996) until the food is consumed, thereby causing disease. Pathogenic *Vibrio* species are a health concern especially in fish harvested from poor quality waters (ICMSF, 1986). The level of these pathogens in shellfish and in water does not correlate with the level of indicator organisms. In addition human pathogenic *Vibrio* species can acquire survival strategies that enable them to evade detection by conventional monitoring techniques (Odeyemi, 2012), necessitating direct detection of each species in order to ensure public health and food safety (Harwood et al., 2004). The level of *Vibrios* in sea water is generally high during summer months, but very low or undetectable during winter when cultural methods of detection are used due to the ability of these organisms to revert into a dormant unculturable state (Whitehouse et al., 2010).

Pathogenic *Vibrio* species associated with seafood food

Vibrio cholerae

V. cholerae is the most commonly occurring pathogenic *Vibrio* species, followed by *V. parahaemolyticus*. Cholera is characterised by profuse watery diarrhoea with flakes and mucus, dehydration and sometimes death when adequate medical intervention is not instituted (Jay et al., 2005; Kaper et al., 1995; Talkington et al., 2011). Huq et al. (1990) have suggested that *V. cholerae* is wide spread in estuarine and marine waters around the world, although the numbers may be low in sea water throughout the year. They are found in areas where salinity is between 4 to 17% and their presence does not correlate with either *E. coli* or *Salmonella*. They establish symbioses with planktons as a means of overcoming low temperatures that prevail during winter in temperate regions (Huq et al., 1990; Montanari et al., 1999). *V. cholerae* is sensitive to temperatures, higher than 45℃, and to many disinfectants used in the food industry.

Studies elsewhere on the survival patterns of *V. cholerae* have shown that the bacteria remained viable for 14 days in refrigerated raw vegetables and at room temperature (28 to 30℃) for 28 days. On dry cereals (maize, rice and biscuits) the survival time was 1 to 5 days at 4℃. On the same cooked food item survival times were 14 and 24 days for room temperature and refrigeration storage respectively. It survives longer under refrigeration conditions, with 4 to 9 days in raw, and 2 to 21 days in cooked fish (ICMSF, 1996). Infections by *V. cholera* can lead to epidemics, pandemics, or may be endemic in specific areas. Since its discovery in 1817 there have been seven cholera pandemics. The bacteria are carried around the world mostly by asymptomatic carriers. The most recent outbreak occurred in Haiti and it was linked to a clone in Nepal (Talkington et al., 2011; Hendriksen et al., 2011).

Vibrio parahaemolyticus

V. parahaemolyticus associated gastroenteritis manifested by profuse, watery diarrhoea free from blood and mucus, abdominal cramps, nausea, and vomiting. Outbreaks of *V. parahaemolyticus* food poisoning are associated with consumption of raw molluscs (Oysters, clams), cooked crustaceans (shrimp, crab and lobsters) in America and Europe, but in Japan, South East Asia, India and Africa raw fish is always implicated as a vehicle (Jay et al., 2005).

V. parahaemolyticus is usually resident in coastal waters (Adams and Moss, 2008), but recently it has been isolated from fresh water fish (Noorlis et al., 2011). It is halophylic requiring 3 to 10% NaCl for growth. It is distinguished from other *Vibrio* species by its inability to ferment sucrose. It is mesophilic hence isolated only in summer in temperate regions, but in warm water (19 to 20℃), it is detected throughout the year (ICMSF, 1 996). It is associated with crustaceans; shrimp and crab, and molluscan shell fish (Popovic et al., 2010) and free swimming fish at a concentration of 10^2 to 10^3 cells per gram, while in very warm water there may be 10^6 cfu per gram (ICMSF, 1996). About 98 of the bacteria isolated from marine animals and seawater are Kanagawa negative and were regarded to be non pathogenic (ICMSF, 1996). However Pal and Das (2010), Rojas et al. (2011) and Jun et al. (2012) showed that up to 35% of 60 *V. parahaemolyticus* bacteria isolated from shell fish and fin fish samples collected from markets, aquatic environments and restaurants carried the Thermostable Direct Haemolysisn (*tdh*) gene some of which were resistant to antibiotics. Velazquez-Roman et al. (2012) confirmed the prevalence of pandemic strains of *V. parahaemolyticus* (O3:K6) in sediment, seawater and shrimp samples from the Pacific coast that coincided with recurrent sporadic cases of gastroenteritis in Northwestern Mexico between 2004 and 2010. During this study 52% of the environmental strains were found to contain virulent genes. *V. parahaemolyticus* is rarely isolated in water where temperatures are below 15℃ (Matches et al., 1971). The bacteria are moderately sensitive to freezing and can persist in frozen food for long periods (Vasudevan et al., 2002). *V. parahaemolyticus* is very sensitive to heat (killed at 47 to 60℃) to ionizing radiation, and to halogens (Adams and Moss 2008). Food poisoning associated with this organism arises from gross mishandling during preparation and from temperature abuse (Nickelson and Finne, 1992).

V. parahaemolyticus has been isolated from shellfish (Bauer et al., 2006; De Paola et al., 2000; Drake et al., 2007; Hervio-Heath et al., 2002; Pal and Das, 2010; Vieira and Iaria, 1993). The levels in oysters correlated with the levels in corresponding waters (De Paola et al., 1990) pointing at the filter feeding habit as the way shell fish pick up the bacteria from waters. *V. parahaemolyticus* was also detected in lobster samples collected from a supermarket and from a fish processing factory at 3 to 21 cfu/g). Some of environmentally isolated *V. parahaemolyticus* were Kanagawa test positive (Vieira and Iaria 1993). The minimum levels of detection for *V. parahaemolyticus* in chilled or raw crustaceans are 10^3/g (Vieira and Iaria, 1993). *V. parahaemolyticus* and *V. vulnificus* have been isolated from bivalve molluscs, but their numbers did not correlate with the number of coliforms (Normanno et al., 2005).

Vibrio vulnificus

V. vulnificus is regarded as an emerging pathogen, infection in humans was first reported in 1964 in the USA and in 1987 in Taiwan (Harwood et al., 2004; Hsueh et al., 2004). It is an opportunistic pathogen in the elderly, immunocompromised or in individuals with impaired liver function, or underlying disease such as cirrhosis, diabetes mellitus or those on steroid therapy (Drake et al., 2007; Harwood et al., 2004). Infections are usually acquired through consumption of raw or improperly cooked shellfish or through contact with seawater (Cazorla et al., 2011; Hsueh et al., 2004). *V. vulnificus* causes three important disease syndromes; septicaemia, necrotising wound infections and gastroenteritis with a mortality rate of 40 to 50% occurring one to two days after onset of the symptoms (Cazorla et al., 2011; Harwood et al., 2004; Hsueh et al., 2004). *V. vulnificus* is a common inhabitant of seawater, but the levels are not correlated with those of indicator organisms (Harwood et al., 2004).Hsueh et al. (2004) have shown that the bacteria are most prevalent in seawater during summer when the temperatures are between 26 to 29℃. It occurs in environments with salinity of 0.5 to 2.5% (Harwood et al., 2004), but the concentration was found to increase in

oil polluted seawater (Tao et al., 2011). *V. vulnificus* is sensitive to low pH's and acid treatment is suggested for effective control of the survival of this pathogen in seafood (Lee et al., 1997). Tamplin and Capers (1992) have demonstrated the presence of *V. vulnificus* (10^3 to 10^5 cfu /ml) in sea water and (10^2 and 10^3 cfu/g) on oyster samples collected from the Gulf of Mexico. They are also found associated with other shellfish (clams, and mussels), sediments and planktons which are believed to act as reservoirs (Harwood et al., 2004). The USA Interstate Shellfish Sanitation Conference has set a limit of 30 *V. vulnificus* per gram oyster (Harwood et al., 2004). Tamplin and Capers (1992) showed that the organisms could not be cleared from the oyster tissues by normal depuration procedures when UV treated and filtered water was used.

Vibrio alginolyticus

Vibrio alginolyticus is largely opportunistic pathogen causing systemic infections in persons with underlying diseases such as the immunocompromised individuals, those with severe burns, cancers or with a history of alcohol abuse (Oliver and Kaper, 1997), though it has occasionally been associated with cases of gastroenteritis and diarrhoea. In healthy individuals *V. alginolyticus* is associated with extra intestinal infections such as wound or ear infections (Novotny et al., 2004). The bacterium was also isolated from the blood of a leukaemia patient alongside *Pseudomonas aeruginosa* (Oliver and Kaper, 1997). *V. alginolyticus* is also important food spoilage organism producing histamine by the decarbolylation of histidine and is responsible for scombroid poisoning characterised by nausea, vomiting, abdominal cramps, neurological disorders and skin irritations (Novotny et al., 2004; Ray and Bhunia, 2008).

V. alginolyticus is the most commonly isolated *Vibrio* species in marine environments from all over the world. Its numbers correlate with increases in temperatures (Oliver and Kaper, 1997). It has been isolated from both fin fish and shell fish. Hervio–Heath et al. (2002) isolated *V. alginolyticus* as the most predominant *Vibrio* species from mussels and water samples from the coastal areas in France. Di Pinto et al. (2006) analysed 38 shellfish samples and detected *V. alginolyticus* from 76% of those samples while only 42% of their samples were positive for *V. parahaemolyticus.* Gonzales–Escanola et al. (2006) and Xie et al. (2005); detected *V. parahaemolyticus* virulence associated genes in some *V. alginolyticus* strains.

Pathogenic strains of *V. alginolyticus* carry the collagenase and ToxR genes and can be identified through dection of these genes (Cai et al., 2009). Detection of *Vibrio* species in food and water typically relied on isolation of the bacteria followed by identification by means of classical biochemical tests (Croci et al., 2007; Harwood et al., 2004). *Vibrio* species are non fastidious and grow readily on basic laboratory media, but some need supplementation of vitamins, amino acids and minerals (Farmer and Hickmann- Brenner, 1991; Thompson et al., 2004). They grow better at alkaline pH (7.5 to 8.5) and require added NaCl. The optimum growth temperature ranges from 15 to 30℃ (Thompson et al., 2004).

Most *Vibrio* species grow on Mac Conkey agar, but do not ferment lactose (Farmer et al., 2004). Isolation of *Vibrio* species from environmental sources usually is done by a pre-enrichment step in Alkaline Peptone Water (APW), pH 8.6 supplemented with 1 to 2% NaCl (Harwood et al. 2004; Kaysner et al. 1992), followed by plating on a solid medium such as thiosulfate citrate bile salts sucrose (TCBS) agar. Enrichment media are normally incubated at room temperatures (18 to 22℃), while solid media are incubated at 25℃. TCBS is a selective differential media that incorporates bile salts, alkaline pH (8.6) and 1% NaCl as selective agents, sucrose as a fermentable sugar and bromothymol blue as the pH indicator (Farmer and Hickmann-Brenner, 1991; Harwood et al., 2004). On TCBS sucrose fermenters form yellow colonies, while non-sucrose fermenters are green (Farmer and Hickmann-Brenner, 1991; Kaysner et al., 1992). Enterobacteriaceae, *Pseudomonas* and Gram positive bacteria are inhibited on TCBS (Harwood et al., 2004). The problems encountered with TCBS are that some species do not grow well on it, the selectivity and performance of the medium may vary from batch to batch or between manufacturers, bacteria other than vibrios may grow on it (Farmer and Hickmann-Brenner, 1991; Harwood et al., 2004; Shikongo-Nambabi et al., 2010) and sometimes TCBS may be too inhibitory for some species (especially *V. vulnificus*). Other media used in the isolation of vibrios include Tryptone Soy Agar to which 1 to 2% NaCl is added and Marine Agar (MA). Luria-Bertani broth is used for the enrichment of psychrotrophic species (Thompson et al., 2004). Lee et al. (1997) showed that Brain Heart Infusion Broth (BHI) was a better enrichment medium for *V. vulnificus* than Luria-Bertani (LB) broth, Cellobiose Polymyxin–B Colistin (CPC) broth or Alkaline Peptone Water (APW).

The media that best suit isolation of *V. vulnificus* from shellfish and other environmental sources include amongst others *V. vulnificus* (VV) agar, CPC agar and its modification, as well as Sodium Dodesyl Sulfate-Polymyxin-B - Sucrose (SPS) agar (Harwood et al., 2004).

Williams et al. (2011) showed that using CPC^+ can help isolate *V. vulnificus* with a high rate of recovery from the environment only when present in high numbers, while CHROMagar *Vibrio* medium can clearly distinguish between four medically important *Vibrio* species. However, the researchers recommended that the two

media be used simultaneously to avoid false positive results.

The viable but non culturable (VBNC) state

The mystery behind the survival of pathogenic *Vibrio species* in sea water that could not be detected by cultural methods at low temperatures was broken when a dormant state of the bacteria was investigated and discovered (Jiang and Chai, 1996; Oliver et al., 1995). This dormant state, termed the Viable but Non culturable (VBNC) state is associated with a number of pathogenic bacteria including *Vibrio* species regulated at the level of gene expression that enhances resistance to stress (Baffone et al., 2006; Wong and Wang, 2004). The VBNC differs from stress responses induced by nutrient limitation (Coutard et al., 2005). Apart from nutrient deprivation low temperature is an essential factor for VBNC state (Coutard et al., 2005; Jiang and Chai, 1996).

The VBNC state is accompanied by alterations in metabolic activities, morphological changes and changes in genes expression. Jiang and Chai (1996) found that starved pathogenic and non pathogenic strains of *V. parahaemolyticus* maintained at low temperatures changed shapes from rods to cocci quicker than the cells that were kept at room temperatures. After one week *V. parahaemolyticus* cells also changed from smooth rod like shapes with a single flagellum to spheroid cells without flagellae, but with polymer like filaments and wrinkled cell walls. Similar morphological changes (rods to cocci) were observed in *V*. *vulnificus* cells kept at 5℃ in ASW microcosms (Smith and Oliver, 2006). Baffone et al. (2006) observed reduced cell size, spherical shapes, formation of blebs and polymer–like filaments in VBNC states whereas no such changes have been reported during stressful conditions at normal growth temperatures.

Alam et al. (2007) showed that after incubation free swimming coccoid *V. cholera* O1 cells in the VBNC could not be cultured, while normal shaped cells recovered from attached biofilms could be grown on culture media. The cell size reduction from rods to cocci is regarded as part of the survival strategy to optimise nutrient uptake and utilisation during the adverse environmental conditions such as those encountered during winters in temperate regions and marine environments (Jiang and Chai, 1996; Wong and Wang, 2004).

Griffitt et al. (2011) using recognition of individual gene fluorescence *in situ* hybridisation (RING-FISH) method showed that culture based methods were unable to detect *V. parahaemolyticus* cells in VBNC hence counts obtained in environmental samples were lower than the actual population density in samples in agreement with Thongchankaew et al. (2011) who used the denaturant gradient gel electrophoresis (DGGE) method to detect *V. prahaemolyticus* in water samples from Tarutao Island, Thailand that could not be detected by cultural methods. Brauns et al. (1991) demonstrated that the (*V vulnificus* haemolysin gene) *vvh* decreased significantly during the VBNC state and was barely detectable using similar conditions that were optimal for detecting the same gene in viable, culturable cells. Oliver and Bockian (1995) demonstrated that though pathogenicity decreased during the VBNC state, *V. vulnificus* strains expressed virulence factors during the dormant state and were able to resuscitate in vivo. Saux et al. (2002) using a Reverse Transcriptase seminested PCR detected the *vvh* gene in VNBC cells induced in Artificial Sea Water (ASW).

Similarly, Coutard et al. (2005) demonstrated that Nonculturable viable *V. parahaemolyticus* cells maintain albeit at reduced levels expression of housekeeping genes (rpoS and 16S-23S rRNA), while the synthesis of virulent associated proteins (Thermostable Direct Haemolysin) was suspended. However, Smith and Oliver (2006) did not detect expression the haemolysin gene (*vvhA*) during the *V. vulnificus* VBNC state induced in ASW microcosms. Using membrane diffusion chambers dipped in the North Carolina estuarine waters however the same researchers (Smith and Oliver, 2006) showed that expression of the *vvhA* was strain dependent. Smith and Oliver (2006) also detected the gene encoding the periplasmic catalase (*katG*); however the levels decreased during the VBNC state and concluded that a decrease of the catalase production triggers cells to adopt the VBNC state since the cells are no longer able to protect themselves against the H_2O_2 that is present in culture media.

Entry into the VNBC may (Coutard et al., 2005) or may not (Oliver et al. 1995) depend on the physiological and nutritional state of the cells. Although Coutard et al. (2005) found that logarithmic phase cells are more readily inducible into the VBNC than stationary phase cells, Oliver et al. (1991) and Oliver et al. (1995) studying *V. vulnificus* observed that both logarithmic and stationary phase cells entered the VBNC state at the same time.

In the VBNC state cells are more resistant to heat, salinity, acid and pH than dividing cells (reviewed by Drake et al., 2007). The resistance of VBNC pathogenic *Vibrio* species to mild treatments is a matter of concern in seafood that is consumed raw or partially cooked Wong and Wang (2004).

The VNBC state is inducible by a temperature downshift to 3 to 4℃ (Coutard et al., 2005; Oliver and Bockian, 1994; Smith and Oliver, 2006; Wong and Wang, 2004) but higher temperatures (10 to 15℃) were req uired to enter this state under natural conditions (Oliver et al., 1995). In both *in vitro* and *in situ* studies viable counts remained high throughout the VBNC state, and the culturability was reversed within 24 h by temperature upshift to 22℃, for a more extensive review, refer to Drake et al., (2007).

Table 1. Phenotypic traits used to differentiate between *V. parahaemolyticus* and *V. alginolyticus* (Farmer et al., 2004; Oliver and Kaper, 1997).

Phenotypic test	*Vibrio parahaemolyticus* (%)	*Vibrio alginolyticus* (%)
Voges-Proscauer (VP) test in1% NaCl	80-95	0
Urea hydrolysis	15	0
Cellobiose	5	3
Dulcitol	3	0
Sucrose	1	99
ONPG	5	0
L- Arabinose	80-89	0-1
Growth in nutrient broth with		
10%	0-2	69-100
12%	0-1	17-100

IDENTIFICATION OF *VIBRIO* SPECIES

General taxonomy

The taxonomy of vibrios was initially based on the classical methods of classification; identification and nomenclature where morphological features (cell shape and presence of extracellular appendages such as flagellae) and biochemical reaction played an important role (Thompson and Swings, 2006). The current taxonomy of vibrios is based on the polyphasic approach that includes phenotypic and molecular methods (Arias et al., 1997; Thompson and Swings, 2006).

The DNA–DNA hybridisation and phylogenetic relationship studies based on the 16S rRNA comparison have been extensively used in *Vibrio* classification (Thompson and Swings, 2006), but with little success in delineating vibrios to species level; most *Vibrio* species have more than 90% 16S rDNA similarities (Aznar et al., 1994).

Multilocus Sequence Analysis (MLSA) is currently used to provide better differentiation of *Vibrio* isolates into respective species (Thompson and Swings, 2006). Although the current family *Vibrionaceae* comprises eight genera the phylogenetic analysis of this group based on the concatenated genes of the three genes, 16S rRNA, *recA* and *rpoA* has proposed four different families e.g. *Vibrionaceae*, *Photobacteriaceae*, *Enterovibrionaceae* and *Salinivibrionaceae* (Thompson and Swings, 2006). The species pathogenic to humans will, however, remain within the genus *Vibrio.*

Phenotypic identification

Phenotypic traits used for the identification of *Vibrio* species are the Gram reaction, oxidase test where vibrios are always positive and the oxidation/fermentation (OF) test in which *Vibrio* species are facultatively fermentative. *Vibrio* species can be differentiated from one another and from *Aeromonas* species by the sensitivity test to the vibriostatic agent O/129 (Famer et al., 2004). Biochemical test systems such as API 20E, and Biolog can also be used for final identification. These methods are, however, slow and unreliable, since some strains exhibit atypical phenotypic characteristics (Thompson et al., 2004). One of the main obstacles is to correctly differentiate *V. alginolyticus* from *V. parahaemolyticus* that have 60 to 70% DNA homology. *V. alginolyticus* was initially classified as a biotype of *V. parahaemolyticus.* The two species can only be differentiated on the basis of a few phenotypic characters (Farmer et al., 2004; Oliver and Kaper, 1997) (Table 1). In addition the two species have have up to 99% 16S rRNA DNA similarity. Determining nucleotide sequences of certain gene such as the hsp60 gave better resolution between the two species (Kwok et al., 2002). A polyphasic approach using phenotypic and molecular biology traits is most reliable, most importantly is the numerical taxonomy and the detection of *V. alginolyticus* species specific collagenase gene (Shikongo-Nambabi et al., 2010).

Immunological based methods

Immunological methods are based on the reaction of antibodies with specific antigens to form immune complexes. Serotyping has been used as a tool for the terminal confirmation during the identification of human pathogenic *Vibrio* species, especially *V. cholerae*. *V. cholerae* is divided into serovars with O1 being the most important and have caused seven cholera pandemics. Strain O139 Bengal is a non O1 strain first isolated in 1992 from the coastal waters of the Bay of Bengal during a cholera epidemic in India, Bangladesh, and in Thailand is also of concern (ICMSF, 1996). The same technique was used in typing the pathogenic strains of *V. parahaemolyticus* O3:K6 and O1: K UT (Iida et al., 2001; Myers et al., 2003). De Paola et al. (2003) used species

specific antisera to differentiate pathogenic strains of *V. parahaemolyticus* into 27 serotypes. Serotyping is of epidemiological significance and can be used to differentiate pandemic from non pandemic strains (Velazquez-Roman et al., 2012).

DNA based methods

Hybridisation

Oligonucleotide probes are used to detect complementary genes or gene fragments as a means of identifying cultures. Probes directed to the variable region of the 16S rRNA gene have been developed, but these were not very useful for *Vibrio* species identification partly due to cross reactions of some probes that reacted with strains other than their specific targets and the specificity of some probes had not yet been tested across the whole *Vibrio* genus. This approach is likely to face problems as some *Vibrio* species may share 100% 16S rDNA homology (Thompson et al., 2004).With the advent of PCR technology the use of hybridisation has largely been replaced.

PCR detection of unique gene fragments

Several methods employing *in vitro* amplification of specific gene fragments by the Polymerase Chain Reaction (PCR) and derivatives of this method have been used for the identification of *Vibrio* species. PCR technique exploits the specificity of short synthetic DNA fragments to bind to complementary sequences and the ability of the DNA polymerase enzymes to directly synthesize the opposite strand under a defined set of conditions using the available DNA as a template. The process is robust, specific and fast hence enabling detection of target genes, gene sequences or specific DNA sequences in test samples.

Hoshino et al. (1998) developed a multiplex PCR consisting of three primer pairs targeting the *rfb*) (gene region specific for O1 and O139) and the cholera toxin (*ctxA*) gene. The *rfb* gene based PCR could detect up to 65 and 200 O1 and O139 cfu per assay in clinical samples respectively. Keasler and Hall (1993) designed a multiplex PCR simultaneously detecting the cholera toxin (*ctxA*) gene in pathogenic and environmental *V. cholerae* O1 Classical and El Tor biotypes and differentiating the two biotypes through their differences in the toxic coregulated pilus (*tcpA)* genes. Theron et al. (2000) developed and evaluated the performance of a seminested *ctxAB* gene specific PCR for the detection of pathogenic *V. cholerae* in environmental water and drinking water sources. This protocol was shown to be highly sensitive, specific and rapid producing results within 10 h. In a quest for a broader spectrum detection protocol due to the fact that non-epidemic strains could also cause disease, the outer membrane protein (*ompW*) gene was targetted. This gene forms part of the *tox*R regulon and was shown to be present in all *V. cholerae* strains and conserved across different biotypes and serogroups, but absent from all other *Vibrio* species studied (Nandi et al*,* 2000). Oligonucleotide primers specific to the *ompW* gene were designed and tested for their ability to amplify the specific gene in both clinical and environmental strains. The specificity of these primers was confirmed using DNA probes (Nandi et al., 2000). Le Roux et al. (2004) have evaluated and identified a combination of three primers for the detection of *V. cholerae ompW* gene in environmental isolates. Their work has shown that the PCR approach is more specific than the API 20E and VITEK 32 systems in identifying environmental *V. cholerae* strains.

Other approaches that have been followed include a multiplex Real Time (RT- PCR) targeting four *V. cholerae* virulence genes (Gubala, 2006) and a similar fourplex Real Time PCR targeting *Vibrio choleare* specific genes, for example, repeat in toxin (*rtxA*), extracellular secretory protein (*epsM*), the toxic coregulated pilus A (*tcpA*) and *ompW* gene with a view to enabling detection of both toxigenic and non toxigenic strains (Gubala and Proll, 2006). More recently Fykse et al. (2007) designed real time nucleic acid sequence based amplification (NASBA) PCR that amplifies specific RNA for a number of virulence factors and house keeping genes. The NASBA detects only actively metabolising cells, as opposed to DNA amplification that might have originated from dead cells.A number of *V. parahaemolyticus* genes have been used as targets to develop species specific PCR based detection. These genes include the thermolabile direct haemolysin, *tl* (Baffone et al., 2006; Croci et al., 2007), the thermostable direct haemolysin, *tdh*, the thermostable direct haemolysin related haemolysin, *trh (*Baffone et al., 2006*)*, the phosphatidyl serine synthetase gene (pR72H fragment) (Lee et al., 1995), gyrase B gene (Venkateswaran et al., 1998), the metalloprotease gene (Luan et al., 2007) and the collagenase gene (Di Pinto et al., 2006). Venkateswaran et al. (1998) cloned and sequenced the gyrase B (*gyrB*) gene of *V. parahaemolyticus* and of its close genetic relative, *V. alginolyticus*. They (Venkateswaran et al., 1998) subsequently developed oligonucleotide primers (Vp-1 and Vp-2r) that amplify a 285bp fragment from the *V. parahaemolyticus gyrB* gene by PCR. All *V. parahaemolyticus* strains were recognised by this primer set, and false positives were not detected. Lee et al. (1995) developed a pair of oligonucleotide sequences (Vp32 and VP33) that bind to opposite ends of a 320-387bp DNA fragment termed pR72H,from the chromosome of *V. parahaemolyticus* that was shown to be found only in this species. This is a fragment of

unknown function located after an rRNA operon and composed of a non coding region and a phosphatidylserine synthetase gene that was found conserved in *V. parahaemolyticus* (Lee et al., 1995; Robert-Pillot et al., 2002). Hervio-Heath et al. (2002) used the *V. parahaemolyticus* (Vp32/Vp33), the *tdh* and the *trh* specific primers to identify suspect isolates and to determine their pathogenicity respectively.

Kim et al. (1999) developed *V. parahaemolyticus* specific primers that detected the species specific Tox–R gene. Low amplification signals were, however, also obtained with closely related species, *V. alginolyticus* and *V. vulnificus*. They (Kim et al., 1999) recommended that detection of this gene as an identification tool should be supplemented with screening the suspect isolates for *V. parahaemolyticus* virulence specific genes (*tdh* and *trh*) so as to confirm the results. Luan et al. (2007) developed PCR primers specific for the *V. parahaemolyticus* metalloprotease gene and used 101 bacterial strains, 85 of which were identified by phenotypic methods as *V. parahaemolyticus* to assess the specificity of their new primers. When the specificity and sensitivity of the new primer *VPM1* and *VPM2* were compared to three other primer sets that were already in use (including primers pairs directed against three other known virulence genes; (*tl, tdh* and *trh*) the designed metalloprotease specific primer pair gave the best results with a sensitivity of up to 4pg DNA. Unlike the L-*tdh*/R-*tdh* and L-*trh*/R-*trh* primer sets that gave false negatives, *VPM1*/*VPM2* detected all the *V. parahaemolyticus* strains tested, and did not react with other bacteria. Reverse transcriptase (R-T) PCR for *tdh, trh1* and *trh2* have also been designed (Mothershed and Witney, 2006).

Di Pinto et al. (2006) used three oligonucleotide primer pairs specific for either *V. parahaemolyticus*, *V. cholerae* or *V. alginolyticus* collagenase gene and have demonstrated a simultaneous detection of the two species (*V. parahaemolyticus* and *V. alginolyticus*) that were present in alkaline peptone water (APW) enriched shellfish tissue homogenates. In addition the researchers recommended use of these primer pairs in discriminating between *V. alginolyticus* and *V. parahaemolyticus*. This multiplex-PCR was able to detect the presence of these two bacterial species in some culture negative samples circumventing the low sensitivity inherent in culturing and the inability of phenotypic tests to identify isolates with atypical biochemical profiles. Qian et al. 2008 cloned two proteins from *V. alginolyticus* (*OmpK* and *OmpW*) expressed them in *E. coli* and designed specific primers to the genes. More recently Dalmasso et al. (2009) designed a multiplex PER (Primer extension reaction) PRC protocol directed against *rpoA* gene to simultaneously detect and identify six human pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *V. fluvialis*) in fishery products. At the same time Cai et al. (2009) designed a multiplex PCR against collagenase, ompK and toxR genes to differentiate virulent and avirulent strains of *V. alginolyticus*. Virulent strains were recognised by the presence of the collagenase and toxR genes that were absent from avirulent strains. The test also improved the rapidity, sensitivity and specificity for *V. alginolyticus* detection and could detect up to 8.8×10^2 cfu.The gyrase B gene was also targeted (Kumar et al., 2006) for *V. vulnificus* identification using the primer set *gyr-vv1* and *gry-vv2*. Arias et al. (1995) developed a highly sensitive nested PCR specific for the 23S rDNA of *V. vulnificus* and Lee et al. (1998) developed another nested PCR directed against the *V. vulnificus* haemolysin gene (*vvh*). Real Time PCR (RT-PCR) was later developed to detect *V. vulnificus* in sea water and oyster tissue homogenates targeting the species specific *vvh* gene (Panicker et al., 2004). Chakraborty et al. (2006) developed a species specific PCR targeting the ToxR gene of the less characterised human pathogen, *V. fluvialis* facilitating a successful differentiation of this pathogen from the closely related *Aeromonas* species.

Molecular typing techniques

Various molecular techniques have been used to type the strains belonging to the various *Vibrio* species. These methods include amplified fragment length polymorphism (AFLP), rapidly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), ribotyping, repetitive extragenic palindromic (Rep) sequences, pulsed field gel electrophoresis, Rep (Enterobacterial repetetive intergenic consensus and extragenic palindromic) -PCR, multilocus sequence typing (MLST) and whole genome sequence typing (WGST). Most of these techniques could also be used for the identification of specific *Vibrio* species; they are important tools in epidemiological investigations and have been recommended for determining the source of virulent strains and their temporal and spatial distributions (Caburlotto et al., 2011; Cazorla et al., 2011; Hendriksen et al., 2011; Ju et al., 2009; Thompson and Swings, 2006).

Sequencing of 16S rDNA

Modern methods of identification that compare nucleotide sequences of group specific genes have gained popularity within the last three decades. The 16S rRNA gene is used for both phylogenetic studies and as a taxonomic marker (Thompson et al., 2005).The whole gene or variable regions of the 16S rRNA gene are sequenced through the use of various primer pair combinations. Ideally comparison of the obtained nucleotide sequences with the sequences available in the

Table 2. Copy numbers of 16S rRNA genes detected in bacteria.

Bacterial species	Number of 16S gene copies	Reference
Bacillus subtilis	10	(Conville and Witebsky, 2007)
E. coli	7	(Conville and Witebsky, 2007)
Salmonella Typhimurium	7	(Conville and Witebsky, 2007)
Clostridium perfringens	9	(Conville and Witebsky, 2007)
Clostridium perfringens	9	(Conville and Witebsky, 2007)
Vibrio cholerae	9	(Conville and Witebsky, 2007)
Nocardia species	3-5	(Conville and Witebsky, 2007)
V. parahaemolyticus	11	(Harth et al., 2007)

GenBank, BCCM or other gene banks facilitates identification of unknown isolates. The 16S rDNA PCR method increases the efficiency of bacterial identification due to its rapid, reproducible nature. This notion was supported by Petti et al. (2005) who showed that the 16S rDNA sequencing was able to correctly identify bacteria including pathogens that had been misidentified by traditional methods. Harris and Hartley (2003) developed a broad range 16S rDNA PCR for use in identifying bacteria isolated from various clinical specimens and compared the results with cultural and serological methods. PCR amplification of the 16S rDNA detected many potentially pathogenic organisms from culture negative samples implying that the 16S rDNA PCR improves the identification process as compared to cultural methods. Complete 16S RNA gene sequences of many V*ibrio* species have been determined and *Vibrio* species 16S rRNA gene specific primers and probes have been developed, as cited by Maeda et al. (2003) who developed a clustering scheme based on *Vibrio* species 16S rRNA gene specific PCR that clustered 46 *Vibrio* species into 16 groups.

There are also several problems associated with the use of the 16S rDNA for identifying bacteria. No universally accepted criteria exist for the required level of homology to delineate isolates of the same species or genus. Some bacteria with different phenotypic characters may share up to 100% 16S rDNA sequences whereas less than 99% 16S rDNA sequence homology has been observed in bacteria belonging to the same species (Harris and Hartley, 2003). In some species multiple heterogenous copies of the 16S rRNA gene operons exist (Case et al., 2007; Pontes et al., 2007) (Table 2). Some closely related *Vibrio* species share up to 99% 16S rDNA sequence homology (Shikongo-Nambabi et al., 2010). It is therefore recommended to combine the 16S rRNA gene sequence with other species specific morphological and physiological characteristics to correctly identify *Vibrio* species (Mienda, 2012). The 16S-23S Intergenic spacer (IGS) region nucleotide sequences complement the 16S rDNA in *Vibrio* species identification (Hoffmann et al., 2010). Using the capillary gel electrophoresis technology to analyse the 16S-23S IGS region nucleotide sequences after PCR quickly generates strain and species specific patterns useful in epidemiological and idenfication of *Vibrio* species.

Also the databases used in sequence comparison for identification are public facilities hence sequences that are incorrectly identified may also be erroneously published and could lead to misidentification of accurately read sequences.

Multilocus sequence analysis (MLSA)

With the problems experienced with the prokaryotic identification schemes (phenotypic traits) and 16S rRNA gene sequences, as well as with the DNA- DNA hybridisation (DDH), multilocus sequence analysis (MLSA) is gaining popularity as a promising taxonomic tool to differentiate between closely related bacterial species. MLSA uses gene sequences from more than one locus, generally of house keeping genes that are widely distributed among bacteria to be studies and have single copy in the genome (Pontes et al., 2007; Thompson et al., 2005). The genes should also be long enough to give sufficient information, but should be of the length that permits easy sequencing (Thompson et al., 2005).

The genetic loci that were initially found suitable in MLSA for taxonomic studies of *Vibrio* species included the 16S rRNA gene, *rpoA* (RNA polymerase alpha subunit) and *recA* (recombinant repair protein) and *pyrH* (uridylate kinase gene) (Thompson et al., 2005). Recently Thompson et al. (2007) have developed an MLSA scheme to distinguish *V. harveyi* from the closely related *Vibrio campbellii* species using seven housekeeping genes; *recA*, *topA (*topoisomerase I), *pyrH, ftsZ* (a cell division protein)*, mreB* (actin like cytoskeleton protein), *gapA* (glyceraldehydes-3 phosphate dehydrogenase) and the *gyrB* DNA (gyrase B gene beta subunit). The resulting 3596 nucleotide long DNA gave a better resolution than when only three genes, *gyrB, reA* and *gapA* were used. MLSA therefore acts as a buffer against mutations and horizontal gene transfer (HGT) problems

associated with the 16S rDNA sequences (Thompson et al., 2005). Whitehouse et al. (2010) coupled Electrospray Ionization Mass Spectrometry to Multi Locus Sequence PCR (PCR/ESI-MS) using 8 primer pairs that react with the whole *Vibrionaceae* family to simultaneously detect, quantify and identify *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* species in sea water and fresh water in Georgia. The novel scheme was robust and highly specific able to discriminate between closely related *V. cholera* from *V. mimicus*.

CONCLUSION

The initial microbiological quality of marine fish is depended upon the microbial load of the marine water where the fish is harvested as governed by the prevailing physicochemical parameters (pH, salinity, nutrient content and temperature). The predominant micro-organisms that persist in the finished products to cause spoilage and or food borne diseases depend on the intrinsic factors of the fish in question; the extrinsic factors of the food processing, storage and distribution environment, the explicit factors of the microbial population, and the processing factors. Bacterial metabolism of the final product can cause significant quality deterioration while the presence of pathogens can be hazardous to human health. Stringent quality assurance regimes are required to ensure optimal seafood safety and shelf life.

The current laboratory methods used in quality assurance and quality control are sometimes ineffective due to low sensitivity or atypical phenotypic or genotypic characters of target organisms. A polyphasic approach that encompasses both phenotypic and genotypic traits is often best for accurate identification of sefood borne pathogens particularly human pathogenic *Vibrio* species. Novel methods such as Electrospray Ionization Mass Spectrometry Multi Locus Sequence PCR or the 16S-23S IGS region nucleotide sequences can be used to enhance the discriminative power of *Vibrio* species identification protocols.

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