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Development and Validation of a PNA-FISH method for *Vibrio* detection

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Work done under the guidance of
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and
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DECLARAÇÃO

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RESUMO

As doenças relacionadas com o consumo de alimentos contaminados constituem um sério problema global de saúde pública e são responsáveis por um grande número de infeções, hospitalizações e até mesmo mortes. A estratégia mais eficaz para controlar e prevenir este tipo de doenças passa por uma identificação precoce de alimentos contaminados a fim de se evitar a entrada destes produtos na cadeia de consumo.

Sabe-se que entre os principais patogénios alimentares estão *Escherichia coli* O157, *Salmonella* spp. e *Listeria monocytogenes*. No entanto, em zonas costeiras, onde o consumo de marisco e peixes é mais frequente, o surgimento de *Vibrio* spp., género de bactérias aquáticas gram-negativas, como principais causadoras de doenças associadas ao consumo destes alimentos tem vindo a aumentar. De entre as espécies de *Vibrio; Vibrio vulnificus, Vibrio choleare e Vibrio parahaemolyticus* destacam-se como sendo as mais importantes no que diz respeito à segurança alimentar.

Técnicas como a de hibridação fluorescente *in situ* (FISH) com recurso a sondas de ácido péptido nucleico (PNA), têm vindo a afirmar-se nos últimos anos com diversas aplicações para a deteção de patogénios alimentares. PNA-FISH é uma técnica de deteção simples, rápida e altamente sensível que envolve a hibridação de sondas marcadas com fluorescência, que têm como alvo regiões conservadas do RNA ribossomal bacteriano. A estrutura da molécula de PNA permite que as sondas se liguem rapidamente aos alvos de DNA ou RNA com melhor estabilidade térmica e uma maior afinidade do que outras moléculas.

O objetivo deste trabalho passou pela otimização das sondas de PNA de maneira a uniformizar as condições de hibridação para uma abordagem multiplex para deteção de *V. vulnificus*, *V. cholerae* e *V. parahaemolyticus* em alimentos. Posteriormente, o meio *Alkaline Saline Peptone Water* (ASPW) foi avaliado como forma de enriquecimento para o crescimento simultâneo de *V. vulnificus*, *V. cholerae* e *V. parahaemolyticus* a partir de amostras alimentares, com o objectivo de encontrar um passo de enriquecimento adequado para a técnica de PNA-FISH.

Após os ensaios de otimização das sondas de PNA chegou-se à conclusão que as condições ótimas de hibridação para as três bactérias eram semelhantes, por isso, uma verdadeira abordagem multiplex seria possível. Contudo a avaliação do meio de enriquecimento, ASPW, não foi conclusiva. A maior parte dos alimentos apresentava contaminação natural com *V. parahaemolyticus;* enquanto as espécies d*e V. cholerae e V. vulnificus* demonstraram ser de difícil crescimento na presença da microflora natural da matriz alimentar.

Palavras-Chave: *Vibrio*, PNA, FISH, Patogénios Alimentares

ABSTRACT

Foodborne diseases and consequently foodborne pathogens are an important cause of human illness worldwide, causing a considerable number of infections, hospitalizations and even deaths. These diseases are associated with the consumption of contaminated food products and, thus, the infection sources and routes of transmission are very diverse. Many efforts have been made in the last decades to prevent and control foodborne diseases; but the most efficient strategy relies in the earlier identification of food contamination in order to prevent the release of contaminated products.

It is known that the main foodborne pathogens are: *Escherichia coli* O157, *Salmonella* spp. *and Listeria monocytogenes*. However, in coastal areas, where the consumption of fish and seafood is more frequent, the appearance of *Vibrio* spp., aquatic gram-negative bacteria, has been increasing as the main cause of diseases associated with the consumption of raw seafood. Among the *Vibrio* species, *Vibrio vulnificus*, *Vibrio choleare* and *Vibrio parahaemolyticus* stand out as being the most threatening with regard to food safety.

Some techniques, such as peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH), have been emerging in the last years with several applications for the detection of foodborne pathogens. PNA-FISH is a simple, rapid, and highly sensitive technique, which involves the use of probes coupled with fluorescence labels, which target conserved regions of the bacteria ribosomal RNA. The structure of the PNA molecule allows the probes to bind quickly to the target DNA or RNA with better stability and affinity than other molecules.

The aim of this work was the probes optimization, to standardize the hybridization conditions for a multiplex approach for the detection of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* in food samples. Later, Alkaline Saline Peptone Water (ASPW) was evaluated as an enrichment medium for the simultaneous enrichment procedure of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus*. After the experiments, similar optimum conditions of hybridization were obtained for the three bacteria; therefore, a multiplex approach would be possible. However, the evaluation of the enrichment step with ASPW, was not conclusive. Most of the food samples presented natural contamination with *V. parahaemolyticus*; while *V. cholerae*, and *V. vulnificus* demonstrated to be the difficult to growth due to the natural microflora of the food matrix.

Keywords: *Vibrio*, PNA, FISH, Foodborne Pathogens.

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LIST OF ABBREVIATIONS

CFU – Colony forming unit;
CT – Cholera toxin**;**
DNA – Deoxyribonucleic acid;
ECDD – European Centre for Disease Prevention and Control;
EFSA – European Food Safety Authority;
ELISA – Enzyme-Linked Immuno Sorbent Assay;
FISH – Fluorescence *in situ* hybridization;
GAPs – Good Agricultural Practices;
HACCPs – Hazard Analysis of Critical Control Points;
ISO – International Organisation for Standardisation;
MPN – Most probable number;
PCR – Polymerase chain reaction;
PNA – Peptide Nucleic Acid;
LNA – Locked nucleic acid;
RNA – Ribonucleic acid;
rRNA – Ribosomal ribonucleic acid;
RT – Real Time;
SDS – Sodium Dodecyl Sulphate Polymyxin Sucrose;
STEC – *Shiga-Toxin* producing *Escherichia coli*;
TCBS – thiossulfate citrate bile salts agar;
TCP – toxin-coregulated pilus;
tdh – thermostable direct hemolysin;
tl – thermolabile hemolysin;
trh – TDH-related hemolysin
USA – United States of America;

1. General Introduction – Foodborne Pathogens and Illness

Nowadays studies into microbial diversity and pathogenicity are receiving a lot of attention, due to its impact on food production and human health (Pedrós-Alió, 2006). Foodborne disease has emerged as an important public health and economic problem in many countries during the last two decades (Rocourt, Moy, Vierk, & Schlundt, 2003). In the United States of America (USA), about 9.4 million cases of foodborne illness each year result in 55,961 hospitalizations and 1,351 deaths (Scallan et al., 2011). As most of these cases are not reported, the true dimension of the problem is unknown (European Food Safety Authority, 2015) (World Health Organization, 2007).

In addition to the public health risk and associated costs, foodborne illness represents a threat for food industry which, in a globalized world, faces very strict demands in terms of food security. Food industry needs to comply with quality regulations that require a close control of the microbiological contamination of the products and the overall production plant. Food industries now distribute their products from all over the world, transforming the food industry towards an interconnected system with a large variety of complex relationships. Therefore, the quality control requirements have become stricter and, so, the implementation of quality assurance systems is nowadays a priority. The three most important generic quality assurance systems in the food sector are: Good Agricultural Practices (GAPs), which involve voluntary audits that verify that food is produced, packed, handled, and stored as safely as possible to minimize risks of microbial food safety hazards; Hazard Analysis of Critical Control Points (HACCPs), which identifies specific hazards and measures for their control to ensure the safety of food; and International Organization for Standardization (ISO), established to promote the development of standardization and related activities in the world with a view of facilitating the international exchange of goods and services (Trienekens & Zuurbier, 2008). Companies around the world are increasingly using standard quality assurance systems to decrease foodborne diseases thus improving the quality and safety of products and production processes (Clay, 2002)

1.1 Major Foodborne Pathogens

Some microorganisms are fundamental for the production of some food products, but others are responsible for its deterioration, spoilage and poisoning (Adams & Moss, 2000). Bacteria and yeasts are, among all, those who usually have more impact on food deterioration. With regard to foodborne diseases, bacteria are undoubtedly the main agents (Banwart, 2012). For instance, according to the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDD) agencies, *Escherichia coli* serotype O157, *Salmonella* spp. and *Listeria monocytogenes* together are responsible for nearly 30% of the total foodborne outbreaks reported in the European Union territory (European Food Safety Authority, 2015). In addition to *Salmonella spp. L. monocytogenes* and *Shiga-Toxin* producing *Escherichia coli* (STEC) O157; *Campylobacter* spp., *Yersinia* spp. and *Shigella* spp., are considered to be the major bacterial foodborne pathogens (Scallan *et al*., 2011). However, in coastal areas, seafood and fish production, other foodborne pathogens gain relevance: *Vibrio* genus members.

Vibrio species are widely distributed in the marine environment and at least 11 species are potentially pathogenic to humans (Chakraborty, Nair, & Shinoda, 1997). These species are usually capable of infecting a wide range of aquatic organisms such as penaeid shrimp (Gomez-Gil et al., 1998), several fish species and molluscs (Romero et al., 2014), and then cause infections in humans. Further details on this genus will be discussed in the next section.

1.2 Genus *Vibrio*

The genus *Vibrio* was named by Pacini in 1854 during his studies on cholera, and it is one of the oldest names for a bacterial genus. Pacini also named the cholera bacillus, which eventually became *Vibrio cholerae*, the type species for the genus *Vibrio* (Farmer Iii & Hickman-Brenner, n.d.).

Vibrio are *Gram*-negative bacteria, straight or curved rods or spirals with size ranging from 1.4 to 2.6 μm long with 0.5 to 0.8 μm width, that are usually motile by means of flagella (Farmer Iii & Hickman-Brenner, n.d.). Fischer (1894) made the important observation that the highest viable counts (plate counting method) were obtained when seawater or 3% (wt/vol) NaCl was included in the nutrient medium (Reichelt & Baumann, 1973) (Pfister & Burkholder, 1965). Nonetheless, the genus can be divided into non-halophilic *Vibrios*, including *V. cholerae*, that are capable to grow in media without no added salt, and halophilic species, which only grows

in media with higher contents of salt. The majority of the *Vibrio* species can be included in the halophilic group.

Phenotypic characterization and identification of the genus *Vibrio* has presented several difficulties due to its high biochemical diversity, and description of several new species has led to a constantly changing taxonomy of the *Vibrionaceae* family (Vandenberghe, Thompson, Gomez-Gil, & Swings, 2003).

Due to intensive genomic characterization and the optimization of the screening for phenotypical characteristics, 63 *Vibrio* species are now described and a few species infect marine animals, particularly if they are stressed (Vandenberghe et al., 2003). In addition, so far only 11 of all *Vibrio* species are known to cause intestinal or extraintestinal human infections or have been isolated from human clinical specimens, including: *Vibrio cholerae* (O1 and O139), *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio mimicus, Vibrio hollisae, Vibrio fluvialis, Vibrio alginolyticus, Vibrio damsela, Vibrio furnissii, Vibrio metschnikovii,* and *Vibrio cincinnatiensis* (Janda, Powers, Bryant, & Abbott, 1988). Among these, the first three species cause the majority of the *Vibrio*-associated human infections. *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* are known as causative agents of seawater-related illnesses, such as seafood poisoning in association with gastrointestinal infections (Lee, Lee, Kim, & Park, n.d.) and infections of wounds and mucous membranes (Høi, Larsen, Dalsgaard, & Dalsgaard, 1998). Bivalve shellfish such as clams, oysters and mussels; are of particular concern because they are able to accumulate these bacteria due to their filter-feeding strategy (Høi et al., 1998). The species of *Vibrio* grow well in neutral to alkaline pH 9.0 and are acid sensitive. The optimum pH range is 8 to 8.8 and the optimum growth temperature is 20 to 37°C (Janda et al., 1988). Water temperatures in either side of this range severely affect the bacterial growth.

1.2.1 *Vibrio parahaemolyticus*

Strains of *V. parahaemolyticus* from a few specific serotypes, probably derived from a common clonal ancestor, have lately become pandemic. The organism is phylogenetically close to *V. cholerae*, the causative agent of cholera (Chowdhury et al., 2000). A number of papers describe the distribution and isolation of *V. parahaemolyticus*, and it is generally accepted that the incidence of *V. parahaemolyticus* is highest in marine and estuarine environments (Su & Liu, 2007b).

Historically, *V. parahaemolyticus* was first isolated in 1950, in Osaka (Japan), from a major food poisoning outbreak traced to ingestion of "Shirasu" (partially boiled juvenile sardines)

(Fujino et al., 1953). Although other routes of transmission have been documented, the consumption of raw or undercooked seafood, particularly shellfish, are the major cause of human infection. The consumption of food infected with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever. Thus this bacterium is recognized as an important foodborne pathogen throughout the world, and the leading cause of human gastroenteritis associated with seafood (Hassanain et al., 2013).

The organism has an extremely short generation time (8–12 min) under appropriate conditions. Although an haemolysin produced by the bacterium is thought to be an important virulence factor, the overall mechanisms of pathogenesis is not well-known. Further studies should extend our understanding of the precise relationship between the disease in the human host and the pathogenicity of *V. parahaemolyticus* strains (Wang et al., 2015).

1.2.2 *Vibrio cholerae*

V. cholerae, was first cultured by Koch in 1882 during an epidemic of cholera in India and Egypt. Strains of *V. cholerae* are natural inhabitants of brackish water and estuarine systems where they may constitute the normal microflora of zooplankton and marine animals, such as shellfish (Colwell, 1994). Recent studies have indicated that the human body environment activates some *Vibrio* genes; therefore the species take a more virulent form in the human body (Mooi & Bik, 1997).

V. cholerae strains are grouped into two defined biotypes (El Tor and classical) and more than 200 serogroups. The El Tor and classical biotypes are differentiated based on biochemical properties and phage sensitivity, whereas serogroup differentiation is based on O-antigen structure.

There are at least 206 known serogroups of *V. cholerae* and only two of them can cause epidemic cholera. Those two serogroups are called serogroup O1 and serogroup O139 (O139 is found only in Asia) (Faruque et al., 2003). Pathogenic O1 and O139 isolates typically encode two critical virulence factors: cholera toxin (CT) and toxin-coregulated pilus (TCP) and can cause epidemic cholera (Yamai, Okitsu, Shimada, & Katsube, 1997). The CT is the most important virulence factor. It leads to ADP-ribosylation of G protein thereby giving rise to increased levels of cyclic AMP within the host cell. As a result, electrolyte imbalance occurs due to a rapid efflux of chloride ions and decreased influx of sodium ions, leading to massive water efflux through the intestinal cells, thereby causing severe diarrhea and vomiting, the

principal clinical signs of cholera (Bharati & Ganguly, 2011). The TCP are long, thin, flexible homopolymers that self-associate to hold cells together in microcolonies and serve as the receptor for the cholera toxin phage. *V. cholerae* uses toxin-coregulated pili to colonize the human intestine (J. Li et al., 2008).

The other serogroups are known collectively as non-O1 and non-O139. These other serogroups can cause a less severe diarrheal disease and does not have epidemic potential (Sharma et al., 1998).

1.2.3 *Vibrio vulnificus*

V. vulnificus is a particular virulent halophilic *Vibrio* that typically can cause two different syndromes in infected patients: (1) overwhelming primary sepsis without an obvious source of infection or (2) infection of a preexisting wound, often accompanied by secondary sepsis (Strom & Paranjpye, 2000). Although other cases of infections have been reported, such as gastroenteritis, pneumonia and meningitis. Both types of infection are associated with consumption of seafood or exposure to marine environments.

The number of people infected worldwide with *V. vulnificus* is low when compared to other *Vibrio*, but it is responsible for a significant percentage of *Vibrio*-related illnesses in the United States (Morris, 1988) (Morris & Black, 1985). Infection usually occurs in immunocompromised patients.

This organism is biochemically similar to *V. parahaemolyticus* except that it ferments lactose, and thus it was initially referred to as lactose-positive *Vibrio.*

The isolation of *V. vulnificus* is hampered by the huge diversity of other bacteria present in marine and estuarine environments where they are present. Besides, most of these bacteria have not been phenotypically characterized and many of them can be confused with *V. vulnificus* (Garcia Moreno & Landgraf, 1997).

Currently, *V. vulnificus* is divided into two distinct biotypes based on phenotypic and host range differences. Biotype 1: strains produce indole and ornithine decarboxylase, exhibit several immunologically distinct lipopolysaccharide (LPS) types, and are typically associated with shellfish colonization and human illness; Biotype 2: *Vibrio* strains are negative for indole and ornithine decarboxylase production, and express a common LPS type.

The need to prevent foodborne illness caused by microorganisms requires their rapid detection and subsequent identification. In other words, there is a need to report microorganisms trough

appropriate instruments and getting prove of their identity to prevent the consumption of contaminated food.

Because of the restricted morphological diversity of prokaryotes and the limited usefulness of cultivation-based methods, correct detection and identification of foodborne pathogens may be hard to accomplish (R. I. Amann, Ludwig, & Schleifer, 1995). In the attempt to solve this problem, some faster and specific identification methods, which can be applied on isolated bacteria or directly in the food, have been proposed. These techniques include mainly immunological and molecular methods. Also, recent progress in fluorescence *in situ* hybridization (FISH), a cultivation independent method for the *in situ* analysis of the composition of microbial communities and their dynamics, came to try to address this challenging task (Wagner, Horn, & Daims, 2003).

The next section will address these different techniques that have been applied to *Vibrio* detection.

1.3 Detection of *Vibrio* species

The detection of pathogens in food and clinical samples is a real need in order to prevent the widespread of an outbreak or to provide the right treatment for the infection (PK Mandal, AK Biswas, K Choi, 2011). While the most traditional approaches, based on plating techniques, are still the gold standard for pathogen identification; their use is limited by the time consuming procedures, low discriminatory power and the risk of erroneous interpretations (R. Amann & Fuchs, 2008a). In the last years culture-independent methods have immerged and are often referred to as “rapid methods”, a subjective term used loosely to describe a vast array of testes that includes miniaturized biochemical kits, antibody and DNA-based tests, and assays that are modification of conventional tests to speed up analysis (Law, Ab Mutalib, Chan, & Lee, 2015).

1.3.1 Culture Techniques

Culture techniques are by far the oldest methods of identification, isolation and characterization of bacterial pathogens. The first time that any pathogenic organism had been isolated and studied was when Robert Koch successful isolated *Bacillus anthracis*, in 1876, which at the time was causing the disease anthrax in cattle (Hudson et al., 2008). Koch began his studies using only nutrient broth cultures and he wasn’t having a reliable way to isolate pure strains of bacteria. Some efforts were done in order to solve this problem and Fanny Hesse was the one

that finally, in 1881, came up with the answer to the problem of obtaining pure cultures. Fanny suggested that instead of gelatin, agar should be used in laboratories as an ideal gelling agent (Hesse & Hardy, 1996). Based on this application, selective and differential media have been since that developed in order to facilitate bacterial isolation and characterization.

Taking into account *Vibrio* strains, it is recommended the use of thiossulfate citrate bile salts agar (TCBS). *Vibrio* spp. produce either yellow or green colonies on TCBS, depending on their ability to ferment sucrose. If the sucrose is fermented, yellow colonies are produced. *V. cholera* produces yellow colonies in TCBS whereas *V. parahaemolyticus* produces green colonies. On the other hand *V. vulnificus* produces green colonies (85%) or yellow colonies (15%) (Lotz, Tamplin, & Rodrick, n.d.). In fact, the international standard for *Vibrio* detection/identification from food samples, ISO 21872:2007 (part 1 [ISO 21872-1:2007]: Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae;* and part 2 [ISO 21872-2:2007]: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio Cholerae)* includes the isolation on the TCBS after a pre-enrichment step **(FIG.1.1)**.

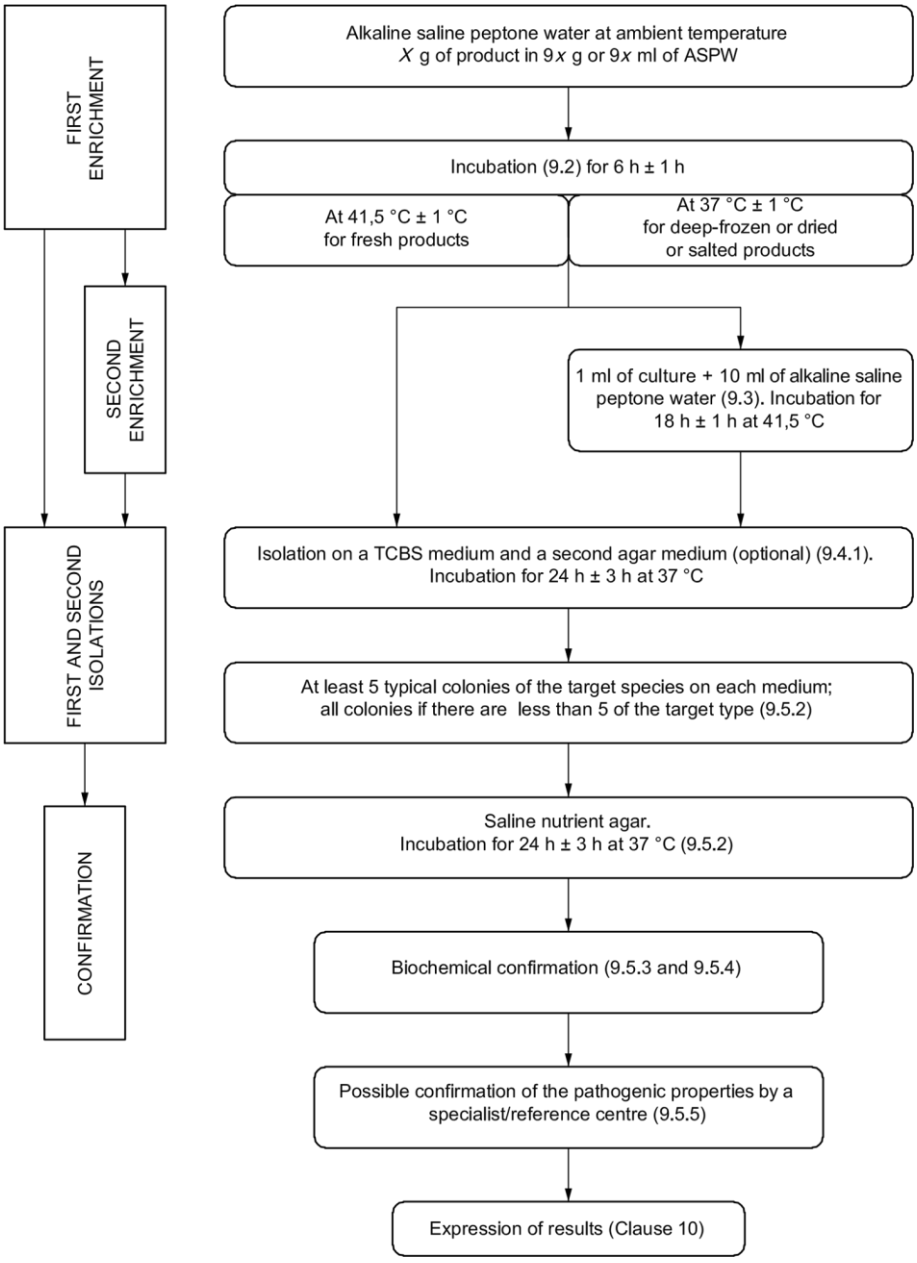

Figure 1.1| Diagram of *Vibrio* isolation procedure according to ISO/TS 21872-1, 2007 (Microbiology of food and animal feeding stuffs – Horizontal method for the detection of potencially enteropathogenic *Vibrio* spp. - Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio choleare*).

Since then, other selective and differential media have been developed for the specific detection of *Vibrio* **(Table 1.1)** and there is now a wide range of options available.

Table 1.1 - Selective and/or differential media for *Vibrio* isolation.

Medium	**Company or Reference**	**Description**
SDS – Agar	HiMedia, India	SDS agar is used for enrichment, isolation and enumeration of *V. vulnificus* from seafood samples.
chromID	Biomérieux, France	Selective chromogenic medium for the isolation of most *Vibrio* species. It particularly enables the presumptive identification of *V. parahaemolyticus* and *V. cholerae*.
CHROMagar *Vibrio*	CHROMagar, France	CHROMagar *Vibrio* medium helps to easily differentiate *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* from other *Vibrio* directly at the isolation step by colony colour.
HiChrome *Vibrio* agar	Sigma – Aldrich, Germany	HiCrome Vibrio Agar is recommended for isolation, and selective chromogenic differentiation of *Vibrio* species from seafood.
***Vibrio Vulnificus* agar (VVM agar)**	(Cerdà-Cuéllar, Jofre, & Blanch, 2000)	In VVM agar, *V. vulnificus* is easily distinguishable from other *Vibrio* strains and other gram-negative bacteria

Nowadays, culture methods also provide the basis for other supplemental tools used in the study of biological bacterial features. These tools can be antigenic and serologic assays, or even other alternative rapid technologies, that can provide additional information on the pathogen identity and profile. Besides, culture is considered an efficient, simple and relatively inexpensive method (Alexander, Deubel, Stephen Dumler, Levine, & Mortimer, 2002).

Despite these advantages, culture has been challenged by recent developed methods such as polymerase chain reaction (PCR). Several studies have shown a superior performance of these new methods over the traditional culture-based techniques (Maurer, 2011). Also, as the rapid detection of food contamination is the most effective way to effectively prevent the release of contaminated products, culture-techniques are being progressively replaced by rapid technologies that can better accommodate the requirements of the quality control systems and public health agencies. A good example of this problematic is observed in Taiwan where 60% of bacterial food poisoning cases occurring between 1991 and 2010 were caused by *V. parahaemolyticus*, because raw seafood are commonly consumed by many Taiwanese (Cheng et al., 2013). This highlight the need for rapid technologies that can identify these bacteria in contaminated food products in a reliable way.

1.3.2 PCR and Variants

There are many DNA-based assay formats, but polymerase chain reaction (PCR)-based methods are the ones that have been developed commercially for detecting foodborne pathogens.

Since its discovery in the 1980s, *in vitro* amplification of nucleic acids using PCR has become a powerful diagnostic tool for the analysis of microbial infections as well as for the analysis of microorganisms in food samples. PCR is a molecular technique based on enzymatic nucleic acid amplification by a heat-stable DNA polymerase (usually known as Taq polymerase). This procedure consists on the hybridization of a pair of complementary oligonucleotides (primers), typically with 20 to 30 nucleotides of length, to a specific region of DNA which will be amplified in a series of 20 to 40 cycles, doubling the amount of sequence copies at each cycle. The result of PCR reaction is then analyzed by an electrophorese procedure, or, for more recent approaches, the DNA amplification is detected in real time due to a fluorescent dye that binds to double strand-DNA. This molecular method has been extensively used as a detection tool for several foodborne pathogens in a number of different samples. They usually target virulence-related or phylogenetic related genes (Hill & Wachsmuth, 2009) (Malorny et al., 2003).

PCR is now gaining a higher acceptance in routine diagnostics, and validated protocols are now emerging. Despite their great advantages, especially in terms of assay time and automation; the complexity of the procedure, which requires specialized technicians, has affected the efficient dissemination of PCR methodology from expert research laboratories to end-user laboratories (Malorny et al., 2003). Also, other disadvantages attributed to PCR include high susceptibility to inhibitory substances, that could provide false negative results; the need for DNA sample purification; susceptibility to cross-contamination and inability to distinguish viable from non-viable cells (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010) (Maurer, 2011). Also, different concentration ratios of the target pathogens/microflora may interfere with the result of PCR approaches (Hsih & Tsen, 2001).

Concerning the application of PCR to the detection of *Vibrio* species, some efforts have been made on this regard. It is known that some genes are useful for the development of *Vibrio*-specific PCR techniques. For instance, thermostable direct hemolysin (*tdh*), thermolabile hemolysin (*tl*), and TDH-related hemolysin (*trh*) genes have been used for detecting the existence of *V. parahaemolyticus* in foods (Bej, Steffan, DiCesare, Haff, & Atlas, 1990). However there may be other genes, like *chiA*, the gene involved in regulation of chitinase expression, thought to be unique in *V. parahaemolyticus* detection (Lin, Lin, Yu, Su, & Tsai, 2016).

While *V. parahaemolyticus* is probably the *Vibrio* species more studied in the genus, PCR applications to detect other relevant species have also been developed (Conejero & Hedreyda, 2004)(Hong et al., 2007).

1.3.3 Immunoassays

The basic principle of immunoassays is the binding of antibodies to a target antigen, followed by the detection of the antigen-antibody complex. In other words, they are used for the quantitative determination of analytes of clinical, medical, biotechnological and environmental significance (Ekins, 1989).

Antibodies are produced by the body in response to a specific invading agent and the most important characteristic of an antibody, when applied to the detection of foodborne pathogens, is its ability to recognize only their target antigen, in the presence of other organisms and interfering food components.

In a direct enzyme-linked immunosorbent or ELISA assays (Engvall & Perlmann, 1971), a solid support is used to immobilize the antibodies, then a test sample is added. Once specific antigen-

antibody bonding takes place, it is common to use another antibody (sandwich format of antigen between two antibodies) conjugated to an enzyme or a fluorescent dye, specific for the antigen. Then the enzyme activity is quantified by using a substrate that produces a colored product or by measuring the emission of fluorescence (O 'sullivan·, Bridges, & Marks, 1979).

Because of the selectivity antigen-antibody, ELISA is superior to other types of immunoassays with respect to sensitivity, specificity and kinetics (Eteshola & Leckband, 2000) . The use of excess capture antibody, enzyme-antibody conjugate, and the chemical-amplification with enzyme-conjugates allows the detection of very low concentrations of analytes.

In 1972, ELISA was used to measure the toxins on *V. cholerae* (Holmgren, 1973) but it was not followed up for bacterial antigens until when Carlsson et al. (1976) assayed antigens from *Brucella*, *Yersinia*, and *Salmonella*.

Studies are being made in order to detect *Vibrio* spp. using ELISA. An example is the production of monoclonal antibodies (mAbs) against TRH of *V. parahaemolyticus* and their application in ELISA to detect pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) from seafood enrichment cultures (Kumar et al., 2011).

1.3.4 Fluorescence *in situ* Hybridization (FISH)

First described in late 1980's (DeLong, Wickham, & Pace, 1989), FISH has become a widely used method for identification, quantification and, in combination with other techniques, characterization of phylogenetically defined microbial populations in complex environments. This technique is based on the annealing of DNA or RNA sequences (usually referred to as probes), attached to a fluorescent label, to a specific target sequence within a cell (R. Amann & Fuchs, 2008a). The most commonly used labels are cyanine (Cy3 and Cy5 are the most widely used) and fluorescein molecules; however, novel generations of fluorophore families are gaining widespread acceptance, which includes for instance the Alexa Fluors and quantum dots. These new dyes bring some advantages like the increased photostability and brightness (Berlier et al., 2003) (Michalet et al., 2005).

rRNA has been refered as the most suitable target for bacterial FISH identification, allowing differentiation of potentially viable cells (B. Li & Chen, 2012). In general, the probes used for FISH identification target the 16S rRNA sequence in members of the *Bacteria* or *Archaea* domain, or the 18S rRNA in *Eukarya* members. The choice of rRNA as a target molecule is related to their abundance in cells. Also, since rRNA is generally the most conserved sequence in all genome (normally used as a phylogenetic marker), it enables the design of

oligonucleotides probes for large taxonomic entities, such as domains, phyla, classes or orders (R. Amann & Fuchs, 2008b) (Stender, Fiandaca, Hyldig-Nielsen, & Coull, 2002).

The ribosome content of microbial cells is variable. In a single cell, rRNA copies range from 100 - 1000 for *Mycobacterium tuberculosis* to 10^4 – 10^5 for *E. coli*. This indicates that the fluorescence intensity and the ribosomal content can be related. Thus, low signal intensity might result from low rRNA content in cells (Pernthaler, Pernthaler, & Amann, 2002).

FISH method usually comprises three main steps – fixation, hybridization and washing. The first one, fixation, stabilizes the cell morphology and permeabilizes the cell membrane, which is important for the hybridization step to take place. In other words, the main objective is to preserve the integrity and shape of all cells and prevent cell loss through lysis; while, on the other hand, permeabilize as many cells as possible to allow the labelled oligonucleotides to diffuse towards their target molecules. The treatment with chemical fixatives such as formalin, paraformaldehyde and ethanol is crucial for an efficient FISH assay (Silverman & Kool, 2007) (Guimarães, Azevedo, Figueiredo, Keevil, & Vieira, 2007).

The following step is the hybridization. In this phase the labelled oligonucleotide probe is added and it is capable to hybridize to its intracellular targets. During this step, temperature, pH, ionic strength and formamide concentrations have to be well define to guarantee the necessary stringency and, thus, the correct hybridization (R. Amann & Fuchs, 2008a).

Finally the washing step ensures that all the excess probe is washed away. The unbound labelled probes are removed from the sample, providing specificity to the detection and a better signal-to-noise ratio. The sample is then ready to single cell identification and quantification by either epifluorescence microscopy or flow cytometry, respectively. The basic steps of FISH are shown in **FIG. 1.2**

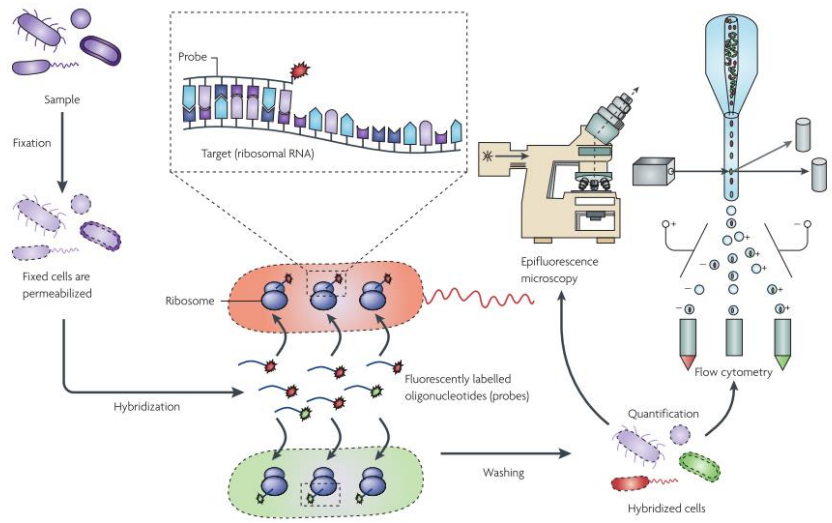

Figure 1.2|Basic steps of FISH. The sample is first fixed to permeabilize the cell membranes and to stabilize the cells. Then, the labelled oligonucleotide probe is added and allowed to hybridize with ribosomal RNA which is the target, in this case. The probe excess is washed away, and the sample is ready for single-cell identification and quantification by either epifluorescence microscopy or flow cytometry (R. Amann & Fuchs, 2008a)

The performance of the probe is an essential factor for the success of the hybridization. Although the recent surge in FISH technology, researchers are aware that not everything is straightforward in the probe design and in the protocol development. For example, cell walls are not always permeable and, because of that, sometimes a pre-treatment with lysozyme or other proteolytic enzymes may be required (L. S. Yilmaz, Okten, & Noguera, 2006). Moreover, ribosome can present a secondary structure that decreases the rRNA accessibility and consequently decreases the hybridization efficiency. The degradation of the probe by proteases or endonucleases of living cells may also represent an obstacle. Furthermore, some probes may not be able to discriminate sequences with single base mismatches, subsequently affecting the specificity (Shakeel, Karim, & Ali, 2006). Therefore, the solution appears to have arrived in a form of a new class of molecules better known as nucleic acid mimics. These molecules are nucleic acids analogues and seem to be able to improve the robustness of FISH methods (Szulc, Wiznerowicz, Sauvain, Trono, & Aebischer, 2006) (Nielsen, Egholm, Berg, & Buchardt, 1991). Several types of synthetic nucleic acid analogues have been developed and applied to FISH

techniques, especially Peptide Nucleic Acid (PNA), 2-O-methyl-RNA and Locked nucleic acid (LNA). Among these we will focus on PNA, which is the nucleic acid mimic more widely applied to FISH technology (Cerqueira et al., 2008).

First designed and published by Nielsen and co-workers and introduced in FISH studies for the detection of microorganisms during the late 1990's, PNA has increased its importance in recent years. This DNA analogue has better hybridization features than the common DNA probes (Prescott & Fricker, 1999) (Drobniewski, More, & Harris, 2000). PNA's, like said before, are synthetic molecules in which the sugar-phosphate backbone is replaced by N-(2-aminoethyl) glycine units, unlike the negative charged sugar-phosphate backbone of DNA (Shakeel et al., 2006). The difference between DNA and PNA probes is shown in **FIG. 1.3**. The nucleobases are practically positioned in the same place and within the same distance as it occurs to the natural DNA. Consequently, PNA can hybridize to negatively charged DNA or RNA without electrostatic repulsion (Cerqueira et al., 2008). The neutral charge of the PNA backbone is perhaps the main reason for its better specify and affinity properties. It contributes to a higher thermic stability of the PNA/DNA duplex when compared to DNA/DNA duplex thanks to its higher melting temperature (Tm).

Melting temperature is an indicator of the PNA/DNA duplex stability (Gracias & McKillip, 2004). Moreover the overall Gibbs free energy change (ΔG) can also be an indicator for the quality of the probe once it measures the affinity of the probe to the target sequence which is lower in PNA/DNA duplex than the DNA/DNA, indicating higher affinity (Armitage, 2003).

The stability of the PNA/DNA duplexes is strongly affected by the presence of imperfect matches. This means that a mismatch in a PNA/DNA duplex destabilizes much more the duplex than a mismatch in a DNA/DNA duplex. For a 15-mer PNA/DNA duplex the average T_m of single mismatch was found to be 15C° compared to 11C° for DNA/DNA duplex (Hyrup & Nielsen, 1996) (Koh & Ph, 1991).

PNA can also hybridize under low salt concentration and, as a consequence, rRNA secondary structure is destabilized and this improve the access of the probe to the target sequence (Cerqueira et al., 2008). Another important benefit, due to the hydrophobic character of PNA probes, is the easier diffusion through the apolar cell membrane (Drobniewski et al., 2000). Therefore, the use PNA-FISH instead of using other traditional techniques, is preferred because it offers a better specificity, stability and quicker binding (Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010). Peptide nucleic acids are the most useful and advanced technology in FISH and the synthesis of custom PNA probes is accessible via a South Korean company named Panagene (http://www.pnagene.com).

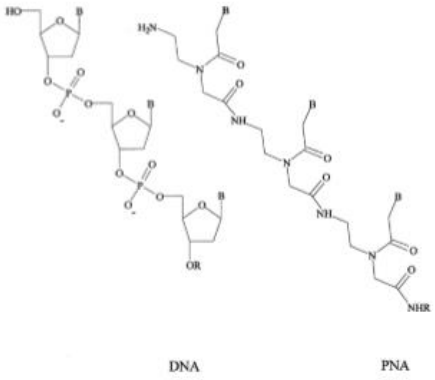

Figure 1.3| Chemical structure of DNA and PNA molecules (adapted from Perry-O'Keefe *et al.*, 2001). PNA probes are oligomers in which single bases are linked by a neutral backbone, this avoid the rejection of negative charges.

Previous studies conclude that PNA-FISH can be an alternative method for rapid identification of *Vibrio* spp. in a broad spectrum of seafood or related samples (Zhang, Li, Wu, Shuai, & Fang, 2015). Therefore, further improvements are still needed to optimize and formulate a universal enrichment medium for better recovery of Vibrio strains from a spectrum of samples having high levels of competing flora. Plus, it is necessary more knowledge regarding PNA-FISH application in *V. parahaemolyticus, V. cholerae* and *V. vulnificus* identification.

2. OPTIMIZATION OF THREE PNA PROBES FOR DETECTION OF *V. PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS*

Three PNA probes, specific for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, have already been designed, but their experimental optimization and evaluation have not yet been performed. Within this chapter the PNA-FISH procedure using these three probes were subjected to several optimization assays.

2.1 Materials and Methods

2.1.1 Bacterial Strains and Culture Conditions

Several reference strains and isolates (*Vibrio* and non-*Vibrio*) were used in the optimization assays and for specificity and sensitivity test. They are listed in **Table 2.6**. Strains of *Vibrio* were maintained into marine agar plates (BD, USA) for 24h at 30°C-37°C (Termaks, Series B8054) and streaked onto fresh plates every 48 hours. Every other strains were maintained in brain heart infusion (BHI) agar (VWR, USA) for 24h at 37°C. For PNA-FISH assays, colonies were streaked in the day before the analysis.

2.1.2 Probes Theoretical Evaluation

Three PNA probes specific for the detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* species, have been previously designed at Biomode by using bioinformatic tools. Those probes were then reassessed to evaluate their theoretical performance before moving with the probes synthesis.

The theoretical specificity and sensitivity of the probe were evaluated with the TestProbe program available in SILVA rRNA database (https://www.arb-silva.de). For this, only rRNA sequence with good quality and with more than 1900 pb were considered. The probes were aligned with a total of 64593 23S rRNA sequences present in the SILVA database. The probes were also tested against the small subunit (16S/18S, SSU) database, on SILVA rRNA database, to evaluate the existence of possible cross-hybridization with the 16S rRNA sequences.

Specificity was calculated as nVs/ (TnV) 100, where nVs stands for the number of non-*Vibrio* strains that did not react with the probe and TnV is the total of non-*Vibrio* strains examined. Sensitivity was calculated as V/(TV)x100, where V stands for the number of *Vibrio* strains detected by the probe and TV for the total number of *Vibrio* strains existent in the databases.
Gibbs free energy (ΔG) was also determined to infer the probes affinity to the target sequence and also to further estimate the predicted Tm. Those values were determined according to Giesen et al., 1998; Yilmaz and Noguera, 2004 (L Safak Yilmaz & Noguera, 2004) (Fontenete, Guimarães, Wengel, & Azevedo, 2015).

2.1.3 FISH Protocol and Microscopy Visualization

The FISH protocol was based on the one described in Almeida *et al.* (2010). Fixation step was done under standard conditions. For optimization of hybridization conditions, a wide range of temperatures (55 to 63°C) and different concentrations of formamide (5,5 and 30%) were tested. After optimization assays the protocol that provide the best results was as follows. Smears of each strain were immersed in 4% (wt/vol) paraformaldehyde (Sigma), followed by 50% (vol/vol) ethanol for 10 min each, and allowed to air dry. The smears were then covered with 20 µL of hybridization solution containing 10% (wt/vol) dextran sulfate (Sigma), 10mM NaCl (Sigma), 30% (vol/vol) formamide (Sigma), 0.1% (wt/vol) sodium pyrophosphate (Sigma), 0.2% (wt/ vol) polyvinylpyrrolidone (Sigma), 0.2% (wt/vol) Ficoll (Sigma), 5 mM disodium EDTA (Sigma), 0.1% (vol/vol) Triton X-100 (Sigma), 50 mM Tris-HCl (pH7.5; Sigma), and 200 nM detecting probe for *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. parahaemolyticus* blocker probe. Samples were covered with coverslips, placed in moist chambers, and incubated for 60 min at 61°C (Termaks, Series B8054). Subsequently, the coverslips were removed and the slides were submerged in a pre-warmed (61°C) washing solution containing 15 mM NaCl (Sigma), 0,1% (vol/vol) Triton X-100 (Sigma), and 5 mM Tris base (pH 10; Sigma). Washing was performed at 61°C for 30 min, and the slides were allowed to air dry. The slides were stored in the dark for a maximum of 24 h before microscopy
For microscopy visualization, the smears were mounted with one drop of non-fluorescent immersion oil (Panreac - AppliChem) and analyzed using a Nikon Eclipse 80i epifluorescence microscope equipped with one filter sensitive to the Cy3 molecule attached to the probes (±550 nm excitation, ±570 nm emission). Other filters present in the microscope that were not capable of detecting the probes fluorescent signal were used in order to confirm that cells did not auto-

fluoresce. For every experiment, a negative control was performed simultaneously for which all steps described above were carried out.

2.1.4 Determination of Experimental Sensitivity and Specificity

The determination of experimental values of sensitivity and specificity of the probe was performed using the bacterial strains in **Table 2.5**. A loopfull of biomass from fresh cultures previously streaked and incubated overnight into Marine agar or BHI plates at 37°C were suspended in 1 mL of sterilized distilled water. A 20 µL drop of suspension was then analyzed following the PNA-FISH protocol described above. Experimental value of sensitivity and specificity was calculated based on an on-line software available on ({ HYPERLINK "http://vassarstats.net/clin1.html" }).

2.2 Results and Discussion

2.2.1 Probe Theoretical Evaluation

V. parahaemolyticus, *V. cholerae* and *V. vulnificus* probes were previously design at Biomode S.A. (**{** HYPERLINK"www.biomode-sa.com" **}**) to target a conserved region in the 23S rRNA region of each one of these three species (**FIG. 2.1**, **FIG. 2.2** and **FIG. 2.3**).

For *V. parahaemolyticus* assays, a blocker probe was introduced because there is only a mismatch between the target sequence of *V. parahaemolyticus* probe and the 23S sequences of other non-*V. parahaemolyticus* strains (**FIG. 2.1**). A blocker probe suppresses the binding of the probe by competition to a non-target site. It hybridizes with non-target sequences preventing those sequences from being available for binding with the detectable probe (Stender et al., 2001). This competition between blocker probe and detection probe ensures that each probe will only bind to highly complementary targets, increasing the specificity of the assay. Nonetheless, it is known that PNA probes are able to discriminate sequences with only one mismatch, however, for optimal discrimination, mismatches should be located in the middle of the probe sequence; which is not the case here. Also, moving the location of the sequence was not an viable option in order to maintain other important mismatch positions. The conserved region for *V. parahaemolyticus* is 5' AGT ATG TGT GGA GCC 3', and is located between positions 2146 and 2161 of *E. coli* 23S rRNA sequence.

For *V. cholerae* probe, a conserved region that allowed a good sensitivity and specificity was successfully identified (**Table 2.1**) (**FIG 2.2**). The target region identified was 5' TCT GAC TAT TGC GAT 3'. This region is located between position 1405 and 1420 of *E. coli* 23S rRNA sequence. Two mismatches are commonly present in the positions 9 and 12 of the probe against other *Vibrio* spp.. With the designed probe is possible to have a false positive result for *Vibrio albensis* strains due to a complete match between the probe and the rRNA sequence.

Finally, for *V. vulnificus* it was not possible to found a suitable conserved region to design a specific probe, however, was identified one region were *V. vulnificus* rRNA sequences are divide into 2 sets, differing in only one nucleotide, 5' CGG AXA CUC UUA AGG 3' Therefore, two different probes were used in order to target the two possible combinations (represented in **FIG 2.3**). The conserved region is located between 1498 and 1513 of *E. coli* 23S rRNA sequence. The position 10 of the designed probes differs in one nucleotide, it can have the configuration of A or G.

Figure 2.1|Target region of the rRNA 23s for *V. parahaemolyticus* probe

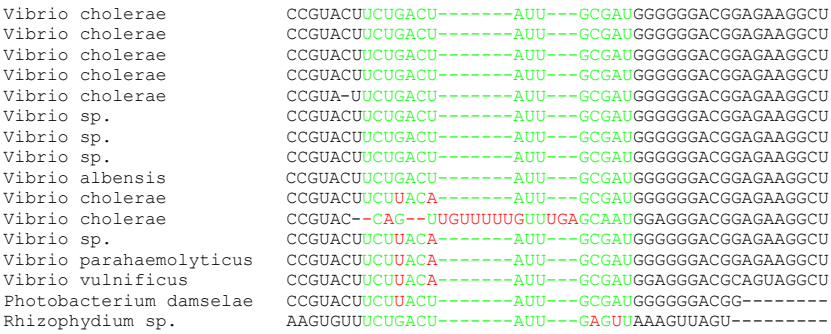

Figure 2.2|Target region of the rRNA 23s for *V. cholerae* probe

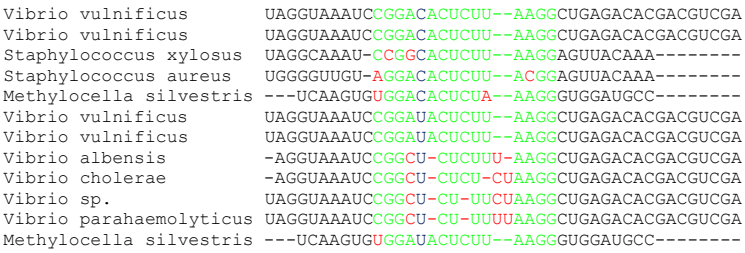

Figure 2.3|Target region of the rRNA 23s for *V. vulnificus* probe

After the evaluation of the target sequences, additional criteria for the selection of the PNA probe included the value of Gibbs free energy (ΔG°) and melting temperatures (Tm).

The value of ΔG° (**Table 2.1**) of the probes is influenced by the composition and the length of the probe. This value should be lower than -13 kcal/mol (Stender et al., 2001). The values of

ΔG° obtained for the probes were between -19,36 and -17,43 kcal/mol. In fact, the values are not within what would be desired, however, is not possible to decrease these values by decreasing the size of the probe, particularly in the case of the *V. parahaemolyticus* probe. To cut position at either the 5' or 3' terminal would put some mismatch positions at the terminal regions, compromising the discrimination power of the probe. Previous studies based on probe target binding have shown that mismatches at the center of the probe have a greater effect on hybridization signals than mismatches close to the ends of the probe (Tomlinson, Harrison, Boonham, Goodchild, & Weller, 2014).

Regarding the predicted Tm (**Table 2.1**), the values obtained ranging between 50,66 - 54,77 ºC. Tm values ranging between 52 - 58 ºC, generally produce better results than probes with lower melting temperatures. On the other hand, melting temperatures, above 65 ºC should also be avoided because of their potential to form secondary structures (Kamel, 2003). The Tm values only differ among them between 1 and 3 ºC, which indicates that a suitable multiplex approach could be achieved.

The predicted/theoretical values of specificity and sensitivity for these probes were calculated as previously described on Almeida *et al*. 2010 (Almeida, Azevedo, Fernandes, Keevil, & Vieira, 2010) (**Table 2.1**). The specificity values were very high ranging from 99,99 to 100%. This means that the designed probe identifies exclusively the bacteria for which it was designed. Regarding sensitivity, values were in most cases close to 100%, the exception was observed for *V. parahaemolyticus* probe that presented a sensitivity of 93.5%. This value is due to the fail in the detection of 12 sequences of *V. parahaemolyticus* in SILVA 23S database. From those sequences, 9 present mismatches with the selected probe and the other 3 may be the result of a fault in the sequencing information.

Table 2.1 – Predicted specificity and sensitivity values for the PNA probes as well as their thermodynamic parameters *Tm* (melting temperature) and ΔG° (Gibbs free energy).

	Probe for *V. parahaemolyticus*	**Probe for *V. cholerae***	**Probe for *V. vulnificus* (A variant)**	**Probe for *V. vulnificus* (G variant)**
Sequence (5'-3')	5' AGT ATG TGT GGA GCC 3'	5' TCT GAC TAT TGC GAT 3'	5' CCT TAA GAG T**A**T CCG 3'	5' CCT TAA GAG T**G**T CCG 3'
Probe name	Vpara_PNA2146	Vcho_PNA1405	VvulA_PNA1498	VvulG_PNA1498
Specificity	100%	99,9%	100%*	100%*
Sensitivity	93,5%	99,3%	100%*	100%*
***Tm* (°C)**	54,15	53,04	50,66	54,77
ΔG°	-19,36	-18,34	-17,43	-18,91

* Sensitivity and specificity values were determined by using the two probes simultaneously.

2.2.2 Determination of optimal probe working conditions

A small set of target and non-target strains were used to determine not only the best signal to noise ratio, but also to confirm the absence of cross-hybridization with related species under the tested conditions. Different hybridization temperatures were used to attest the best hybridization conditions for the three probes. While low temperatures can allow non-specific probe target binding, high temperatures promote denaturation thus impairing hybridization. Formamide was used as a denaturant. it destabilizes the rRNA secondary structure, also improving the probe accessibility (Stender et al., 2002). Thus, formamide is commonly used with the purpose of lowering the temperature at which the hybridization is performed. Formamide concentration at 30% is the most commonly used concentration in PNA-FISH procedures (Zhang et al., 2012).

As such, one of the three PNA probes (for *V. parahaemolyticus*) was first tested at different temperatures and at 30% of formamide concentration **(Table 2.2)**. All the assays were performed with a blocker probe in a proportion of 1:1 (detection probe: blocker probe). This ratio was selected because of the good results (absence of nonspecific signal) obtained by the Biomode researchers faced with similar experiments. The best conditions were verified using temperatures near 61°C. Based on these results the optimization performed for the other probes was accomplished using hybridization temperatures around this value.

Table 2.2 – Hybridization results for *V. parahaemolyticus* probe using different temperatures. hybridization solution included 30% of formamide and a proportion of 1:1 of *V. parahaemolyticus* detecting probe:blocking probe.

	Temperatures				
Strain	**55°C**	**57°C**	**59°C**	**61°C**	**63°C**
V. parahemolyticus MB66	++	++	++	++	++
V. parahemolyticus M37	++	++	++	++	++
V. parahemolyticus MB47 A	++	++	++	+ -	-
V. vulnificus M33	-	-	-	-	-
V. cholerae MB23	-	-	-	-	-
V. harveyi ATCC 1117	++	-	++	-	-
V. fischeri DSM 507	-	-	-	-	-

Regarding the *V. vulnificus* probe, the results show 61°C as the best hybridization temperature. The increase of the hybridization temperature, seems to decrease the sensitivity **(Table 2.3).**

Table 2.3 – Hybridization results for the *V. vulnificus* probe using different temperatures. Hybridization solution used included 30% of formamide.

Strain	**60°C**	**61°C**	**63°C**	**64°C**	**65°C**
***V. vulnificus* CCM 2840**	++	++	++	-	++
***V. vulnificus* M33**	++	++	++	+/-	++
***V. parahaemolyticus* DSM 27657**	-	-	-	-	-
***V. cholerare* MB 27**	-	-	-	-	-
***V. cholerae* MB 23**	-	-	-	-	-
***V. metschnikovii* L1**	-	-	-	-	-
***V. anguillarum* M74**	-	-	-	-	-
***Grimontia hollisae* M106**	-	-	-	-	-
***V. algynolyticus* M17**	-	-	-	-	-

V. cholerae probe was the last one to be evaluated. Three different temperatures, were tested. At 61°C and 60°C the probe demonstrated to be specific. At 63°C the probe signal was weaker **(Table 2.4)**. Therefore, the chosen temperature for the following experimental tests was 61°C

once at this temperature all the probes demonstrate to specific (**Annex IV**). It should be noticed that for the three probes to work well together in a multiplex experiment, similar hybridization condition should be used.

Table 2.4 – Hybridization results for the *V. cholerae* probe using different temperatures. Hybridization solution used included 30% of formamide.

Strain	60°C	61°C	63°C
***V. metschnikovii* M48**	-	-	-
***V. anguillarum* M74**	-	-	-
***V. cholerae* MB27**	++	++	+
***V. cholerae* MB23**	++	++	+
***V. cholerae* MB1**	++	++	+
***V. parahaemolyticus* M37**	-	-	-
***V. parahaemolyticus* MB47A**	-	-	-

2.2.3 Determination of experimental Specificity and Sensitivity for the PNA probes

The determination of experimental sensitivity and specificity of the PNA-FISH procedure was performed using pure culture suspensions. In here a total of 59 strains (**Table 2.5**) were tested including 5 strains of *V. cholerae*, 9 strains of *V. parahaemolyticus* and 5 strains of *V. vulnificus*. The number of strains tested should be as much extended as possible and include phylogenetic-related strains. *Vibrio* strains belong to the *Proteobacteria* Phylum, *Gammaproteobacteria* Class and *Vibronaceae* Family. These strains are ubiquitous in the marine environment, associated with a wide range of marine life.

Of the Proteobacteria Phylum stands out Gammaproteobacteria Class which includes a wide variety of wellknow foodborne pathogens such as *Escherichia*, *Yersinia*, *Shigella* and many other notable genera (Gupta, 2000). As such, non-*Vibrio* strains included in the experiment belong to the same Class than *Vibrio* strains and were chosen with the purpose of testing Gram-negative bacteria phylogenetically close to the target bacteria.

Using 61°C as hybridization temperature, the results show that the three probes are able to detect the strains that they were designed for. The non-target strains have not shown any signal for none of the three probes.

Table 2.5 – Results of the *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* probes specificity and sensitivity test.

ID number	Strain	PNA-FISH output		
		V. cholerae	V. vulnificus	V. parahaemolyticus
CNCTC 7400	*Alivibrio fisheri*	-	-	-
*****	*Vibrio fisheri*	-	-	-
CCM 2580	*Vibrio harveyi*	-	-	-
*****	*Vibrio harveyi*	-	-	-
CCM 2840	*Vibrio vulnificus*	-	++	-
CCM 2838	*Vibrio vulnificus*	-	++	-
CCM 2839	*Vibrio vulnificus*	-	++	-
*** M33**	*Vibrio vulnificus*	-	++	-
*** INSA**	*Vibrio vulnificus*	-	++	-
*** M56**	*Vibrio cincinnatiensis*	-	-	-
*** M44**	*Vibrio fluvialis*	-	-	-
*** M51**	*Vibrio furnissii*	-	-	-
*** M30**	*Vibrio mimicus*	-	-	-
*** M81**	*Vibrio navarrensis*	-	-	-
*** M102**	*Vibrio orientalis*	-	-	-
*** MB1**	*Vibrio cholerae*	++	-	-
*** MB23**	*Vibrio cholerae*	++	-	-
*** MB27**	*Vibrio cholerae*	++	-	-
*** MB84**	*Vibrio cholerae*	++	-	-
*** INSA**	*Vibrio cholerae*	++	-	-
*** MB3**	*Vibrio parahaemolyticus*	-	-	++
*** M37**	*Vibrio parahaemolyticus*	-	-	++
*** INSA**	*Vibrio Parahaemolyticus*	-	-	++
*** MB2023**	*Vibrio parahaemolyticus*	-	-	++
*** MB52**	*Vibrio parahaemolyticus*	-	-	++
*** MB81**	*Vibrio parahaemolyticus*	-	-	++
*** MB47A**	*Vibrio parahaemolyticus*	-	-	++
*** MB65**	*Vibrio parahaemolyticus*	-	-	++

* **MB66**	*Vibrio parahaemolyticus*	-	-	++
* **MB12**	*Vibrio metschnikovii*	-	-	-
* **MB68**	*Vibrio metschnikovii*	-	-	-
* **MB72**	*Vibrio metschnikovii*	-	-	-
* **M48**	*Vibrio metschnikovii*	-	-	-
* **Vb278**	*Vibrio crassostrea/gigantis*	-	-	-
* **Vb150**	*Vibrio crassostrea/gigantis*	-	-	-
* **Vb199**	*Vibrio atypicus*	-	-	-
* **Vb 295**	*Vibrio breoganii*	-	-	-
* **Vb304**	*Vibrio breoganii*	-	-	-
* **Vb219**	*Vibrio gallaecicus*	-	-	-
* **Vb340**	*Vibrio gallaecicus*	-	-	-
CECT 600	*Vibrio alginolyticus*	-	-	-
* **M17**	*Vibrio alginolyticus*	-	-	-
* **M74**	*Vibrio anguillarum*	-	-	-
CECT 522	*Vibrio anguillarum*	-	-	-
CECT 911	*Listeria monocytogenes*	-	-	-
CECT 938	*Listeria monocytogenes*	-	-	-
SGSC 2470	*Salmonella enterica* serv. Dublin	-	-	-
SGSC 2476	*Salmonella enterica subsp. Enteritidis*	-	-	-
ATCC 13525	*Pseudomonas fluorescens*	-	-	-
CECT 832	*Escherichia coli*	-	-	-
CECT 4783	*Escherichia coli*	-	-	-
ATCC 19835	*Shigella dysenteriae*	-	-	-
SGSC 5429	*Yersínia enterocolitica*	-	-	-
ATCC 11296	*Klebsiella pneumoniae*	-	-	-
*	*Pseudoalteromonas*	-	-	-
DSM 10	*Bacillus subtilis*	-	-	-
CECT	*Bacillus thuringiensis*	-	-	-
*	*Bacillus cereus*	-	-	-
*	*Penibacillus larvae*	-	-	-

* isolates

The experimental specificity and sensitivity values of the probes are shown in **Table 2.6**

The results show 100% specificity and 100% sensitivity for the three probes. To be sure of the experimental performance of the probe, additional target strains should be used. Although there were used several non-target strains for the specificity test, the sensitivity test should be

performed with a higher number of target-strains to increase the confidence levels. As it is possible observe in **Table 2.6**, the low confidence level for sensitivity values is quite low, a problem that can be solved by increasing the panel of strains tested.

Table 2.6 – Experimental values of specificity and sensitivity for the *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* PNA probes.

	V. parahaemolyticus	***V. cholerae***	***V. vulnificus***
Specificity	100%	100%	100%
Sensitivity	100%	100%	100%
95% confidence Interval (sensitivity)	[63%;100%]	[46,2%;100%]	[46,2%;100%]
95% confidence Interval (specificity)	[91,1%;100%]	[91,7%;100%]	[91,7%;100%]

Based on these results, the constitution of a multiplex approach for the specific detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, seems possible to be achieved as probes have presented satisfactory sensitivity and specificity values. Optimal conditions of hybridization for these three pathogens are similar so far. Using 61 °C, as hybridization temperature, 30% of formamide percentage and blocker probe in a proportion of 1:1 (detection probe: blocker probe) for *V. parahaemolyticus*, an effective discrimination of these three species is achieved.

3. ENRICHMENT STEP OPTIMIZATION AND VALIDATION IN FOOD MATRICES

The first objective of this thesis was to optimize *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* PNA probes, in order to further establish optimal hybridization conditions for a multiplex PNA-FISH approach targeting *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in food samples. The second objective was to evaluate different enrichment procedures, in order to select a suitable one for application in the PNA-FISH methodology.

The enrichment step is crucial when doing food testing assays. It will provide the right nutrients to the growth of a specific target species, or group of microorganisms, that can be present in low concentrations or have a slower growth. This allows balanced concentrations of the target pathogens to be achieved on a short period of time.

In this chapter the most commonly used enrichment broth for *Vibrio* spp. was evaluated. Then the selected enrichment conditions were tested in food matrix.

3.1 Growth parameters of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in the enrichment medium using different types of peptones

There are two main standard methods used for the detection of *V. parahaemolyticus, V. cholerae and V. vulnificus*. The first one known as ISO standard (International Organization for Standardization), ISO21872:2007 (Microbiology of food and animal feeding stuffs — Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp.) is divided in two parts. The Part 1 (ISO 21872-1:2007, Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*) is used for *V. parahaemolyticus* and *V. cholerae* detection; while Part 2 (ISO 21872-2:2007, Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*.) is used to detect other species than *Vibrio parahaemolyticus* and *Vibrio cholerae*. ISO is an independent, non-governmental organization, and its members are the standards organizations of the 162 member countries. It is the world's largest developer of voluntary international standards and facilitates world trade by providing common standards between nations. Nearly twenty thousand standards have been set covering everything from manufactured products and technology to food safety, agriculture and healthcare (www.iso.org).

The second standard method widely usually used is a FDA (Food and Drug Administration) recommended method. FDA sets scientific standards for testing foods for various contaminants. BAM (Bacteriological Analytical Manual) is the FDA's preferred laboratory procedures for the detection in food and cosmetic products of pathogens (bacterial, viral, parasitic, plus yeast and mold) and of microbial toxins. In the case of *Vibrio* detection, BAM method described at "Chapter 9 – *Vibrio",* is followed.

Laboratories and food companies worldwide use these standards to make sure that food products are safe to eat and drink. Alkaline Saline Peptone Water (ASPW) is designated by the both standard methods as the primary broth used for the pre-enrichment step of *Vibrio* species. ASPW is composed by peptone and salt (NaCl) both at 1% and 2% for BAM and ISO, respectively.

ASPW was first formulated by Shread, Donovan and Lee, (Shread, Donovan, & Lee, 1981) to be used as a non-selective enrichment broth for the cultivation of *Aeromonas* species. Cruickshank reported that the raised pH of the medium could be used to effectively cultivate *Vibrio* species (Cruickshank, 1968). The medium exploits the ability of *Vibrio* species to tolerate alkaline conditions and high sodium chloride levels (Shread et al., 1981) (Croucher, Houston, Bayliss, & Turner, 1983). Regarding salt (NaCl), which is necessary for the growth of *Vibrio* strains, its content in ASPW was standardized to 1% and was latter increased to 2% to allow better growth of the marine *Vibrios* (Ottaviani, Masini, & Bacchiocchi, 2003). If no NaCl is added, it becomes much more selective for *V. cholerae* and *V. mimicus* (non-halophilic species) (Farmer Iii & Hickman-Brenner, n.d.).

As referred before, in the ISO 21872-1, ISO 21872-2 and BAM methods for *Vibrio*, test samples are incubated in ASPW which is composed by salt, peptone and water; however, none of these two methods specifies the peptone used in the medium. Additionally it is known that all types of peptone are used in the composition of broths to be used in enrichment procedures, and the different peptones are known to have a different effect on bacterial growth (Heidemann et al., 2000). Peptones are excellent natural sources of amino acids, peptides and proteins in growth media and used as a carbon source. Even though the problem of unknown complex ingredients in media, such as peptone, is being recognized as a significant factor in media performance; very little work has been done on the performance of different sources and batches of peptones (Gray, Müller, Watkins, & Lloyd, 2008).

Peptones usually are obtained by enzymatic digestion or acid hydrolysis of natural products such as animal tissues, milk, plants or microbial cultures. There are an enormous number of peptones and extracts which can promote and sustain the growth of most organisms. Therefore,

four different types of peptones: soy peptone, meat peptone, yeast extract and casein peptone were tested.

Meat peptones are made from different types of tissues. Heart, lungs, esophagus, skin and bones are the most commonly used (Gray et al., 2008). The biochemical composition of the constituents of these tissue types differ and consequently affect the performance of the medium with respect to their support for microbial growth.

Other peptone made from animal sources is casein. Peptones belonging to the casein group were diverse in their ability to support bacterial growth (Gray et al., 2008).

Soy peptones, belonging to the group of plant peptones, result from the hydrolysis of plant proteins and are considered to sustain better cell viability and longevity compared to some other hydrolysates.

Finally, yeast extract usually produced the best performance of all the peptones as it should contain all the amino-acids required for bacteria growth (Gray et al., 2008) (Michiels et al., 2011).

This experiment intends to evaluate the type of peptone which is capable to provide the best conditions for the growth of these strains as well as the best concentration to work with.

3.2 Materials and Methods

3.2.1 Bacterial Culture

The bacterial strains used throughout this work were *V. parahaemolyticus* (MB47 A), *V. cholerae* (MB23) and *V. vulnificus* (CCM 2838). The strains were grown in Marine Agar at 30 ºC for 18h. A loop-full of previously grown bacteria was diluted in phosphate-buffered saline (PBS 1x: NaCl 137mM, KCl 2,7mM, Na2PO4 10mM, KH2PO4 1,8 mM). Then the optical density (OD) of the suspension was adjusted to 0,1 (corresponding to 10^8 cells/mL - a calibration curve was previously performed to correlate the OD with the bacterial concentration in PBS, **Annex III**.) Finally, bacteria suspensions were further diluted in PBS (10 fold dilutions) to obtain the desired concentration for inoculation, and used directly or placed in the refrigerator (± 5 ºC) for 48h, for the experiment with stressed cells. Bacteria concentration was confirmed by plating 100 µL of each dilution sample on Marine Agar in triplicate.

3.2.2 Determination of Growth Parameters

Four different types of peptones were used: casein (Merck, German), yeast extract (Merck, German), meat peptone (Merck, German) and soy peptone (Merck, German). Two concentrations for each peptone were evaluated, 1% and 2% (wt/vol). The four peptones named before were then mixed with 1% (wt/vol) and 2% (wt/vol) of NaCl and adjusted to a final pH of 8,6 ± 0,2. Optical density (OD) growth curves were obtained using a Sinergy H1 (BioTek, USA) microplate reader and data analysis was performed in the GEN5 (BioTek, USA) software. The assay was performed using a 96-well plate, with each well containing peptone medium (270 µL) and bacterial culture (30 µL). Negative controls, which consisted in 300 µL of peptone medium without bacterial culture at 37 ºC, were used to define the base line. Each condition was run in triplicate, the OD was measured every one hour for 60 h at a wave length of 600 nm applying a correction factor, available at the software. The plates were shaken for 10 s before each measurement.

The obtained OD's, after the removal of the respective negative control, were plotted as a function of time and the growth rates were obtained using the points that compose the exponential phase. A straight line was fitted to those points and the slope was calculated as the specific growth rate for each condition. The lag phase was considered to be the time it takes for the OD to increase from the base line.

3.3 Results and Discussion

Regarding determination of growth parameters in different formulation of ASPW, four different types of peptone (meet, soy, casein and yeast) were evaluated for three different strains (*V. parahaemolyticus* MB 47A, *V. cholerae* MB 23 and *V. vulnificus* CCM 2838). The experiment was performed with stressed cells (after cold exposure) and with non-stressed cells (without cold exposure).

The purpose of using stressed cells, in the first series of experiments, was because cells are usually stressed in normal conditions, whether for low or high temperatures or by competition with other strains, cells are usually stressed. So, using stressed cell the experiment would be closer to the real conditions. However, injured cells become sensitive to inhibitory agents in specific selective media and hardly grow and produce colonies (Bissonnette, Jezeski, McFeters, & Stuart, 1975). Thus, in the second series of experiments, non-stressed cells were used in order to establish a comparison between growth parameters of both stressed and non-stressed cells even knowing that indeed the best conditions, were obtained with non-stressed cells.

Growth rate curves were obtained (**Annex I and Annex II**) for each condition in triplicate and based on that curves, lag time and specific growth rate were then calculated. The results obtained are shown in **Table 3.1 and Table 3.2**.

Growth parameters for cold exposed cells

Regarding the cold exposed cells **(Table 3.1)**, in general and as expected, the growth rate values were lower and the lag phase values were higher than those with not stressed cells.

The length of the lag phase is an important factor to take into account. It is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium (Rolfe et al., 2012). A short lag phase indicates that the adaptation to the media by bacteria was fast and efficient thus bacteria starts exponential phase faster.

Beginning with *V. parahaemolyticus* strain, for which higher growth rate values were verified, the best growth conditions were obtained at a concentration of 2% (wt/vol) of salt and meat peptone (0,2969 h^{-1}) and 2% (wt/vol) of salt and soy peptone (0,2848 h^{-1}). In general, with values ranging from 0,0516 to 0,1369 h^{-1} for 1% (wt/vol) of peptone and salt concentrations, and values ranging from 0,1574 to 0,2969 h^{-1} for 2% (wt/vol) peptone and salt concentrations.

The lag phase values obtained for this strain ranged between 2 and 5 h. The highest lag phase values (5 h) were obtained for salt and soy peptone at both concentrations 1% and 2% (wt/vol). *V. cholerae* demonstrated to grow in all peptones at both concentrations. The values were quite similar, ranging from 0,0369 to 0,0797 h^{-1} for 1% (wt/vol) of peptone and salt concentrations and values ranging from 0,0463 to 0,0914 h^{-1} for 2% (wt/vol) peptone and salt concentrations. In fact, for 1% (wt/vol) salt and peptone concentrations, the values demonstrated to be lower than 2% (wt/vol) salt and peptone concentrations. All the lag phase values reached 3h for all the *V. cholerae* conditions, excluding the value obtained for salt and soy peptone at 2% (wt/vol) which was 6 h.

Regarding *V. vulnificus*, the strain did not grow at salt and casein 2% (wt/vol) and salt and soy peptone at 1% (wt/vol). The values obtained for this strain were better for salt and meat peptone at 2% (wt/vol) (0,0689 h^{-1}) and for salt and yeast extract at 2% (wt/vol) (0,0668 h^{-1}). The lag phase values obtained were the highest among the three strains, as with the non-stressed *V. vulnificus* cells. The values ranged between 5 to 8 h, excepted for salt and soy peptone at 1% (wt/vol), which was 34 h.

In what concerns to the lag phase, as expected, stressed cells have a higher lag phase than non-stressed cells, which indicate that after a cold exposure the *Vibrio* strains have difficulty in adapting to the medium conditions. The values of the lag phase are not very different, except for *V. vulnificus*. This strain has the highest lag phase values in either stressed or non-stressed cells.

Table 3.1– Growth Parameters of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, after a cold exposure, in ASPW with different peptones at different concentrations.

Bacteria	Condition	μ (h^{-1})*	σ**	Lag time	Lag time σ
***V. parahaemolyticus* MB47 A**	Meat/NaCl 1%	0,0832	0,0095	3	0
	Casein/NaCl 1%	0,0516	0,0027	3	0
	Soy/NaCl 1%	0,1026	0,0026	5	0
	Yeast/NaCl 1%	0,1369	0,0029	3	0
	Meat/NaCl 2%	0,2969	0,0229	2	**0**
	Casein/NaCl 2%	0,1900	0,0000	2	0
	Soy/NaCl 2%	0,2848	0,0034	5	0
	Yeast/NaCl 2%	0,1574	0,0143	2	0
	Meat/NaCl 1%	0,0608	0,0010	3	0
	Casein/NaCl 1%	0,0369	0,0040	3	0
	Soy/NaCl 1%	0,0491	0,0035	3	0

***V. cholerae* MB 23**	Yeast/NaCl 1%	0,0797	0,0032	3	0
	Meat/NaCl 2%	0,0622	0,0100	3	0
	Casein/NaCl 2%	0,0463	0,0026	3	0
	Soy/NaCl 2%	0,0914	0,0005	6	0
	Yeast/NaCl 2%	0,0594	0,0043	3	1,4
***V. vulnificus* CCM 2838**	Meat/NaCl 1%	0,0213	0,0002	6	0,47
	Casein/NaCl 1%	-	-	-	-
	Soy/NaCl 1%	0,0169	-	34	0
	Yeast/NaCl 1%	0,0405	0,0027	8	0
	Meat/NaCl 2%	0,0689	0,0090	7	0
	Casein/NaCl 2%	0,0280	0,0000	5	0
	Soy/NaCl 2%	-	-	-	-
	Yeast/NaCl 2%	0,0668	0,0019	7	0

*μ: specific growth rate; **σ: standard deviation;

Growth parameters for cells without cold exposure

Regarding the non-stressed cells **(Table 3.2)**, it can be observed that there is a significant difference between the two concentrations tested. The values of the specific growth rate for 1% (wt/vol) of peptone are lower than those for 2% (wt/vol). In general, values ranging from 0,0405 to 0,1125 h^{-1} were obtained for 1% (wt/vol) of peptone and salt concentrations, and values ranging from 0,1133 to 0,2047 h^{-1} for 2% (wt/vol) peptone and salt concentrations.

For *V. parahaemolyticus* the best growth conditions were obtained at a concentration of 2% (wt/vol) of salt and meat peptone (0,2047 h^{-1}) and 2% (wt/vol) of salt and soy peptone (0,2003 h^{-1}).

V. cholerae demonstrated to grow well in all peptones, excluding casein, at both concentrations. The values were quite similar, ranging from 0,0763 to 0,1356 h^{-1} for 1% (wt/vol) of peptone and salt concentrations and values ranging from 0,1250 to 0,1704 h^{-1} for 2% (wt/vol) peptone and salt concentrations. In fact, for casein, the values demonstrated to be lowest: 0,0567 h^{-1} for 1% (wt/vol) of casein peptone and salt concentrations and 0,0768 h^{-1} for 2% (wt/vol) of casein peptone and salt concentrations.

On the other hand, *V. vulnificus* demonstrate to have the worst performance between the three strains. This strain did not grow in casein and salt 1% (wt/vol) and soy and salt 2% (wt/vol), and seems to grow better in meat and yeast extract both at 2% (wt/vol). Thus, the best growth values obtained for *V. vulnificus* are 0,1547 h^{-1} for yeast extract and salt at 2% (wt/vol) and 0,1429 h^{-1} for meat and salt at 2% (wt/vol)

Regarding the lag phase values, the results obtained are different for stressed cells and for non-stressed cells.

For *V. parahaemolyticus* the values of the lag phase ranging between 1 and 3 h, therefore the best value (1 h) was obtained for 2% (wt/vol) meat peptone and salt concentrations. *V. cholera* demonstrate to have similar values of lag phase (between 2 and 3 h) except for casein and salt at 2 % (wt/vol) concentrations where the value has reached 4 h.
Regarding *V. vulnificus* strain, the values of the lag phase were the highest among the three strains. The worst results were obtained for soy and salt at 1 % (wt/vol) and yeast and salt at 1% (wt/vol), the values were 19 and 25 h respectively.

Table 3.2– Growth Parameters of *V. parahaemolyticus, V. cholerae* and *V. vulnificus,* without a cold exposure, in ASPW with different peptones at different concentrations.

Bacteria	Condition	μ (h^{-1})*	σ**	Lag time	Lag time σ
***V. parahaemolyticus* MB47 A**	Meat/NaCl 1%	0,0701	0,1106	2	0
	Casein/NaCl 1%	0,0405	0,0027	2	0
	Soy/NaCl 1%	0,1125	0,0053	3	0
	Yeast/NaCl 1%	-	-	-	-
	Meat/NaCl 2%	0,2047	0,0042	1	0
	Casein/NaCl 2%	0,1133	0,0097	2	0
	Soy/NaCl 2%	0,2003	0,0107	3	0
	Yeast/NaCl 2%	0,1863	0,0152	2	0
***V. cholerae* MB 23**	Meat/NaCl 1%	0,0904	0,0071	2	0
	Casein/NaCl 1%	0,0567	0,0033	2	0
	Soy/NaCl 1%	0,0763	0,0027	3	0
	Yeast/NaCl 1%	0,1356	0,0041	2	0
	Meat/NaCl 2%	0,1250	0,0229	2	0
	Casein/NaCl 2%	0,0768	0,0053	2	0
	Soy/NaCl 2%	0,1704	0,0085	4	0
	Yeast/NaCl 2%	0,1279	0,0097	3	0
***V. vulnificus* CCM 2838**	Meat/NaCl 1%	0,0354	0,0022	2	0
	Casein/NaCl 1%	0,0186	0,0002	2	0
	Soy/NaCl 1%	0,0553	0,0073	19	0,47
	Yeast/NaCl 1%	0,0661	0,0310	25	0,94
	Meat/NaCl 2%	0,1429	0,0133	2	0
	Casein/NaCl 2%	0,0582	0,0012	2	0
	Soy/NaCl 2%	-	-	-	-
	Yeast/NaCl 2%	0,1547	0,0058	5	0,47

*μ: specific growth rate; **σ: standard deviation;

Based on these results the chosen peptone was meat peptone at a concentration of 2% (w/v) and NaCl at a concentration of 2% (w/v) as, in general, these conditions provided the best growth rates and lower lag phase for both stressed and non-stressed cells.

4. DETECTION OF *V. PARHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS* IN MUSSEL

After choosing the best conditions [meat peptone and salt at 2% (wt/vol)], an enrichment step using ASPW at 37°C was performed, in order to evaluate the presence of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* in food matrices. For this purpose, 25g of a food matrix (mussel) artificially contaminated with different levels (1, 10 and 100 CFU) of each one of the strains, were used to simulate real conditions. Three replicates of each level were performed and the enrichment procedure was carried out up to 24h.

It was also executed a second individual assay in which ASPW composition was adjusted in order to formulate a selective growth condition. For instance, *V. vulnificus* is a lactose-fermenting *Vibrio* and *V. cholerae* is a sucrose-fermenting *Vibrio.* Based on this, the second used medium was formulated with a percentage of lactose or sucrose, peptone and salt in order to understand if the presence of this sugars in the metabolism of the strains was important to the specific growth in the enrichment step.

4.1 Materials and Methods

4.1.1 Enrichment Step Optimization

For the optimization of the enrichment procedure, ASPW was selected at a concentration of 2% meat peptone and 2% of NaCl. In order to simulate real enrichment conditions, a food matrix was used. In this case, mussels were used and acquired in local retailers (Pingo Doce and Continente, Braga).

For artificially contamination of mussels samples, inoculums of *V. parahaemolyticus* MB 47A, *V. cholerae* MB 23 and *V. vulnificus* CCM 2838 were prepared from fresh overnight culture in marine agar. Cells were then suspended in a phosphate-buffered saline (PBS 1x: NaCl 137mM, KCl 2,7mM, Na2PO4 10mM, KH2PO4 1,8 mM) solution and adjusted to a cell density corresponding to approximately 10^8 cells/ml. Cells were further diluted in PBS to obtain the desired cell concentration for inoculation into 25g of mussel sample with concentration ranging from 1 to 100 CFU/25 g of food. Cell concentrations were confirmed by plating 100 µL of each

dilution on marine agar. Twenty-five g of artificially-contaminated broken raw mussels were mixed with 225 mL of ASPW in sealed stomacher bags with filters (VWR International, USA). Those assays were repeated with a new formulation of ASPW that included 1,8 g/L of sucrose (ASPW+S) for *V. cholerae* assays and 1,8 g/L lactose (ASPW+L) for *V. vulnificus* assays. The remaining formulation included 2 g/L of NaCl and 0,2 g/L of meat peptone at a pH of 8,6 ± 0,2. The enrichment was performed as described above. A non-inoculated food sample was included for each experiment to check for any possible natural contamination with *V. parahaemolyticus, V. cholerae* and *V.vulnificus.*

4.1.2 Detection of *V. parahaemolyticus, V. cholerae* and *V.vulnificus* in Mussels by PNA FISH

After an overnight pre-enrichment (16 to 24 h) at 37°C in ASPW, 20 µL samples were taken and placed directly in the microscope slide. All samples were dried (approximately 5 min at 61°C), and then hybridization was performed as described above (Chapter 2).

A culture confirmation test was done according to ISO 21872-1:2007 by plating approximately 10 µL of the pre-enrichment samples on TCBS (VWR International, USA). chromID (bioMérrieux, France) agar was also used to confirm the species presence. For TCBS, *V. cholerae* ferment sucrose, which results in a pH shift and production of yellow-brown colonies. On the other hand *V. parahaemolyticus* will produce light bluish colonies and *V. vulnificus* green (85%) or yellow (15%) colonies. Once plated in chromeID, colonies of *V. cholerae* are bluish-green to green, colonies of *V. vulnificus* are bluish-green to blue and colonies of *V. parahaemolyticus* are pink.

4.2 Results and Discussion

The results are show in **Table 4.1** and it can be observed a decrease of the inoculation levels for all the strains, after cold exposure. This was expected since cold causes loss of viability in *Vibrio* strains (Oliver, 1981). The results show positive results for control samples (which are non-inoculated) for the assays performed with *V. parahaemolyticus.* This happens because mussels are susceptible to natural contaminations with this species. Studies mention that this organism is frequently isolated from a variety of raw seafood, particularly shellfish (Su & Liu, 2007a), thus it was impossible to evaluate the effect of the enrichment procedure as the initial concentration of pathogen is unknown. Although natural contaminations levels are not known, *V. parahaemolyticus* was easier to detect at 24h, for all the contamination levels.

In opposition, *V. cholerae* growth was only detected by FISH in one of the three assays, at 16h for a detection level of 100 CFU. For the lower levels, no positive results were achieved even for the longer incubation periods of 24h.

V. vulnificus is indeed the hardest strain of the three to obtain results with, as previously verified in **Tables 3.1 and 3.2** through the lag phase and specific growth rates. No grow was observed in ASPW for any of the inoculation levels even for 24h.

Table 4.1 – Matrix assays results for artificially inoculated mussels samples. *V. parahaemolyticus* MB 47A, *V. cholerae* MB23 and *V. vulnificus* CCM 2838 were used for inoculation and samples enriched for 16 and24 hours before the PNA FISH evaluation.

	Theoretical inoculation level	**Inoculation level /25g (before cold exposure)**	**Inoculation level /25g (after cold exposure)**	**Control***	**16h**			**24h**		
***V. parahaemolyticus* MB 47A**	100 CFU	21 CFU	/	++	/	/	/	++	++	++
	10 CFU	7,5 CFU	/		/	/	/	++	++	++
	1 CFU	0,5 CFU	/		/	/	/	++	++	++
***V. cholera* MB 23**	100 CFU	20 CFU	11,5 CFU	-	-	-	-	-	-	-
	10 CFU	1,5 CFU	0,5 CFU		-	-	-	-	-	-
	1 CFU	0,5 CFU	0 CFU		-	-	-	-	-	-
V. vulnificus	100 CFU	26 CFU	0 CFU	-	-	-	-	-	-	-

CCM 2838	10 CFU	2,5 CFU	0 CFU		-	-	-	-	-	-
	1 CFU	2 CFU	0,5 CFU		-	-	-	-	-	-
***V. parahaemolyticus* MB 47A**	100 CFU	113 CFU	23,5 CFU	-	-	-	-	++	++	++
	10 CFU	14,5 CFU	3,5 CFU		-	+	+	-	+	+
	1 CFU	5,5 CFU	0,5 CFU		-	-	-	-	-	-
***V. cholera* MB 23**	100 CFU	27 CFU	29,5 CFU	-	+	+	+	-	-	-
	10 CFU	5,5 CFU	4,5 CFU		-	-	-	-	-	-
	1 CFU	0 CFU	0,5 CFU		-	-	-	-	-	-
***V. vulnificus* CCM 2838**	100 CFU	29,5 CFU	/	-	-	-	-	-	-	-
	10 CFU	4,5 CFU	/		-	-	-	-	-	-
	1 CFU	0,5 CFU	/		-	-	-	-	-	-

/ - not performed

* Controls were performed for each time point evaluated, but no variation was found between the different time points.

After the results obtained, a second assay was performed and ASPW composition was adjusted in order to try to formulate a selective growth condition.

Selective media are used to limit the level of competing microflora within the sample that may interfere with the growth of the target pathogen. In this case, the addition of sugars was used with the purpose of being used as a selective compound (Harwood, Gandhi, & Wright, 2004) (Finkelstein, 1996). For instance, *V. vulnificus* is a lactose-fermenting *Vibrio* and *V. cholerae* is a sucrose-fermenting *Vibrio*, unlike *V. parahaemolyticus* who is unable to metabolize those two sugars. Taking this information into account and in order to take advantage of this metabolic variety, it was used a variation of ASPW. The content in peptone was lowered and was added instead lactose or sucrose, respectively for the enrichment of *V. vulnificus* and *V. cholerae.*

The results are shown in **Table 4.2**.

Table 4.2 – Matrix assays results for mussels samples enriched in ASPW, ASPW-S and ASPW-L artificially inoculated with *V. parahaemolyticus* MB 47A, *V. cholerae* MB23 and *V. vulnificus* CCM 2838 respectively. Samples were enriched for 18 and 24 before the PNA FISH evaluation.

	Theoretical evaluation level	**Inoculation level /25g**	**Control***	18h			24h		
***V. parahaemolyticus* MB 47A**	100 CFU	/	-	/	/	/	/	/	/
	10 CFU	/		/	/	/	/	/	/
	1 CFU	1 CFU		++	++	++	++	++	++
***V. cholera* MB 23**	100 CFU	25,6 CFU	-	-	-	-	-	-	-
	10 CFU	5 CFU		-	-	-	-	-	-
	1 CFU	0,3 CFU		-	-	-	-	-	-
***V. vulnificus* CCM 2838**	100 CFU	32,3 CFU	-	-	-	-	-	-	-
	10 CFU	3,3 CFU		-	-	-	-	-	-
	1 CFU	0 CFU		-	-	-	-	-	-

/ - not performed

* Controls were performed for each time point evaluated, but no variation was found between the different time points.

This strategy was not successful as it can be seen in **Table 4.2**. The addition of lactose didn't potentiate the growth of *V. vulnificus* neither the addition of sucrose potentiated the growth of *V. cholerae*, as initially predicted. The low concentration of peptone used, 0,2 g/L, may have led to this outcome.

Regarding *V. parahaemolyticus* using standard ASPW, **Table 4.2**, the results show that this strain is detectable by PNA-FISH, with a inoculation level of 1 CFU/25 g after 18 h of enrichment.

Regarding *V. cholerae* and *V. vulnificus*, previous studies demonstrated that an overnight enrichment with ASPW at pH 8.6 should be enough for the detection of *Vibrio*, however, for these strains, some investigators recommend two successive enrichments (Huq et al., 2012).

The presence of a high amount of microflora in mussels allied to a high lag phase value, previously demonstrated in **Tables 3.1 and 3.2**, can possibly be hindered the growth of *V. cholera* and *V. vulnificus* up to detectable levels by PNA-FISH.

It is also known that mussels are reservoirs of opportunistic pathogenic microorganisms to aquatic organisms and humans (Gu & Mitchell, 2002). *V. parahaemolyticus* is part of the natural microflora of mussels, however, other bacteria such as *Aeromonas media, A. veronii, A. salmonicida subsp. salmonicida, and Shewanella putrefaciens* are also present and, such as *V. parahaemolyticus*, are foodborne pathogens (Gu & Mitchell, 2002). The presence of a wide variety of microflora in mussels arise from his filter-feeding capacity and may be the cause to the recurrent contamination of *V. parahaemolyticus.*

As an alternative of addition of sugars, other substances, such as Sodium Dodecyl Sulphate (SDS) and Polymyxin B sulphate, might be added to the enrichment broth, once they are used in SDS agar (HiMedia, India) for differential isolation of *V. vulnificus* and *V. cholerae* (www.himedialabs.com). The medium contains proteose peptone and beef extract which provide necessary growth nutrients like nitrogenous and carbonaceous compounds. Bromothymol blue and cresol red act as pH indicators. Sodium dodecyl sulphate and polymyxin B sulphate are the selective agents. Since the biochemical reaction of *V. vulnificus* on SDS agar appears to be due to a fairly specific straight-chain sulfatase, this differential and selective medium may elicit an important biochemical characteristic in the rapid identification of isolates of *V. vulnificus* (Bryant, Jarvis, & Janda, 1987).

5. GENERAL CONCLUSIONS AND FUTURE WORK

This work had two major objectives. The first involved the optimization of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* PNA probes, in order to further establish optimal hybridization conditions for a multiplex PNA-FISH approach targeting *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in food samples. The second goal involved the selection of an enrichment broth for the simultaneous enrichment of the three pathogens, suitable for PNA-FISH analysis.

Regarding the first aim, from the results it can be concluded that *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* PNA probes are specific and sensitive probes for their target bacteria. Also, the optimal hybridization conditions obtained were similar for the three PNA probes, which indicates that a constitution of a multiplex approach for the specific detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, seems in theory possible, since hybridization conditions are now defined.

Concerning the selection of a suitable enrichment medium for PNA-FISH analyses, it was described that ASPW provide the best growth conditions on the simultaneous enrichment of the three pathogens (ISO/TS 21872-1, 2007). However, this medium was unable to promote the growth of *V. vulnificus* and *V. cholerae* until detectable levels by PNA-FISH, at least in a 24 h enrichment period.

In an attempt to increase the levels of *V. cholerae* and *V. vulnificus* the addition of specific sugars, lactose and sucrose, that are consumed by these bacteria was evaluated. The addition of lactose and sucrose in the constitution of ASPW did not promote the specific growth of *V. vulnificus* and *V. cholerae* as initially expected. Although mussels were acquired in different local retailers, the majority of the samples were natural contaminated with *V. parahaemolyticus* strain which difficult the enrichment step optimization.

Besides the enrichment step is not satisfying so far, it might be possible that different sugar concentrations or the addiction of other selective compounds can be tested to further improve the enrichment step.

Following, some other suggestions of future and complementary works are presented based on the results obtained within this work. Further tests might include:

- Testing of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* probes in a wide range of strains to increase the confidence levels of specificity and sensitivity values obtained so far;

- Construct a multiplex approach of the three pathogens using three probes simultaneously and evaluate if the sensitivity and sensitivity values remain unaltered;

- Use different food matrices besides Mussels;

- Use of other simultaneous enrichment approaches such as double-step enrichment, selective compounds and techniques of sample concentration to increase *V. cholerae* and *V. vulnificus* concentration until detectable level.

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ANNEX I – CALCULATION OF THE GROWTH PARAMETERS OF *V. PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS* IN ASPW WITH DIFFERENT PEPTONES AFTER COLD EXPOSURE

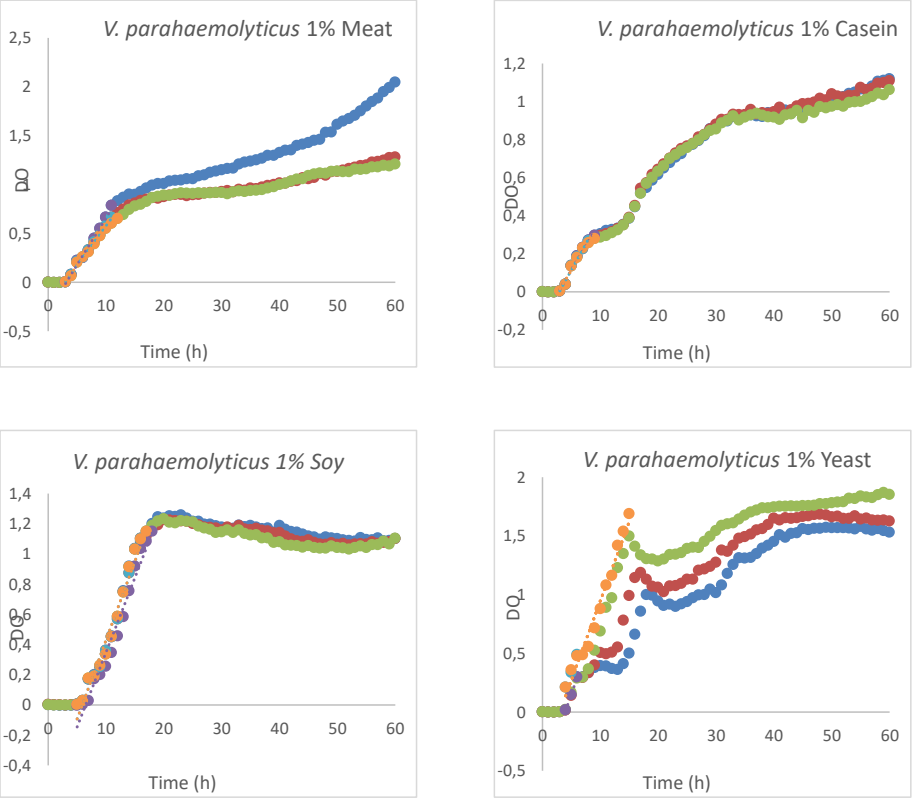


Figure 1: Growth curves for cold stressed V. parahaemolyticus cells at 1% (wt/vol) of both salt and peptone. Four types of peptone: meat, casein, soy and yeast, were used.

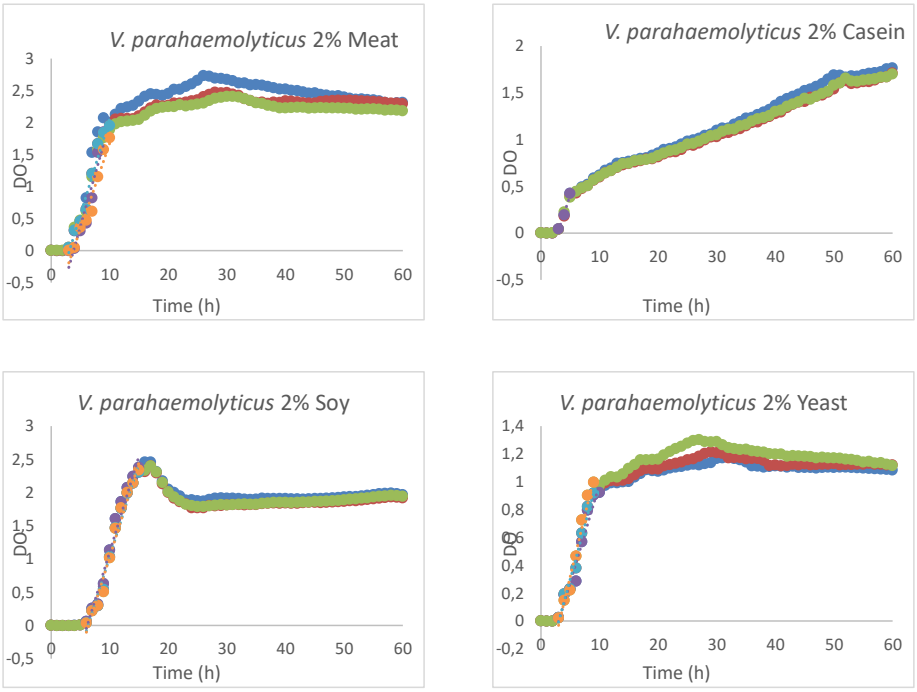


Figure 2: Growth rate curves for cold stressed V. parahaemolyticus cells at 2% (wt/vol) of both salt and peptones. . Four types of peptone: meat, casein, soy and yeast, were used.

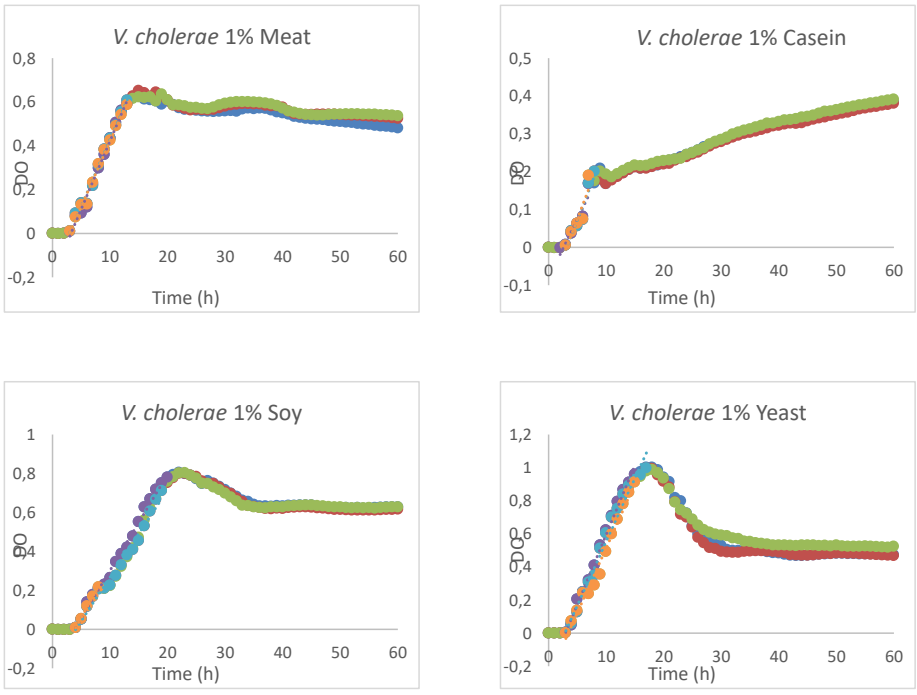
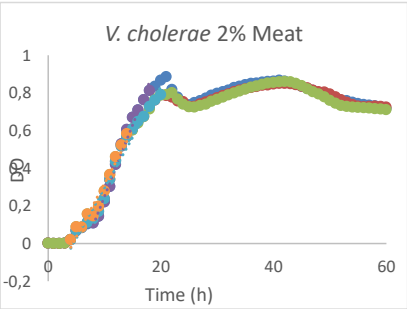
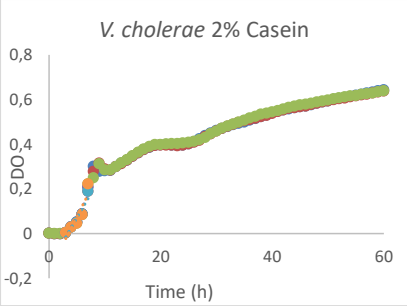
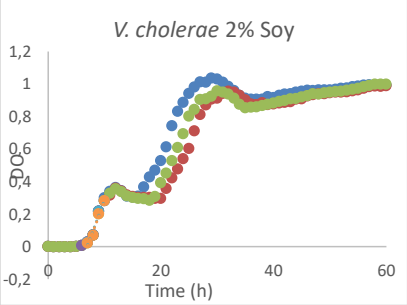


Figure 3: Growth rate curves of stressed V. cholerae cells at 1% (wt/vol) of meat, casein, soy and yeast peptone and salt







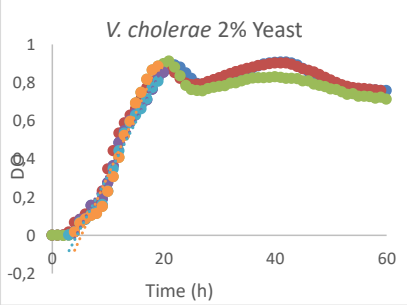
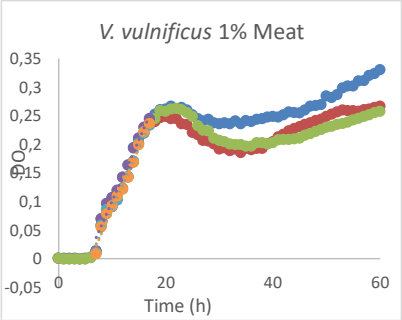
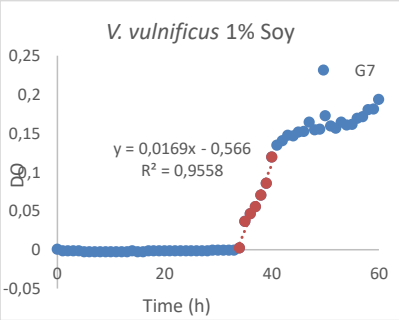


Figure 4: Growth rate curves for cold stressed V. cholerae cells at 2% (wt/vol) of both salt and peptones. Four types of peptone: meat, casein, soy and yeast, were used.





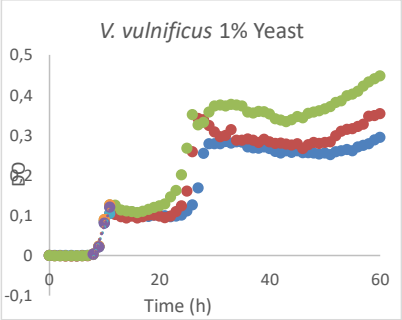
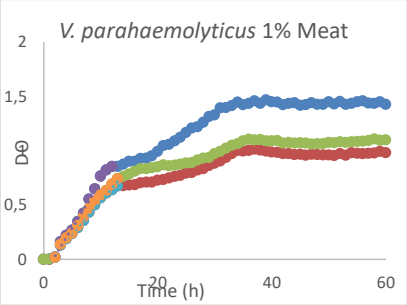
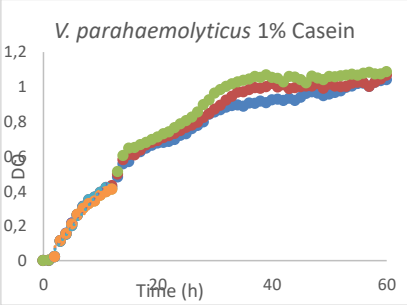


Figure 5: Growth rate curves for cold stressed V. vulnificus cells at 1% (wt/vol) of both salt and peptones. . Four types of peptone: meat, casein, soy and yeast, were used.

ANNEX II – CALCULATION OF THE GROWTH PARAMETERS OF *V. PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS* IN ASPW WITH DIFFERENT PEPTONES WITHOUT COLD EXPOSURE





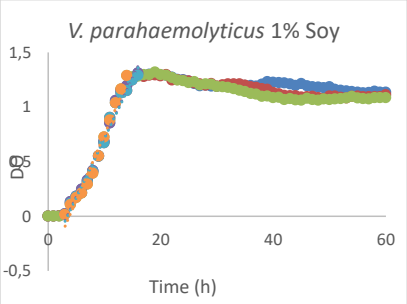
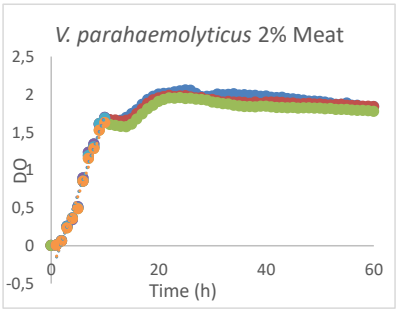
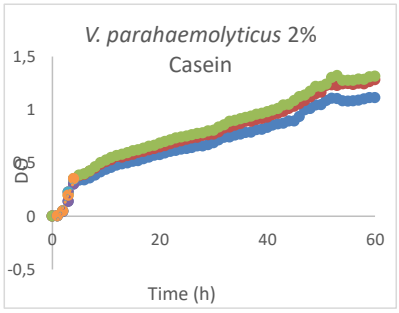
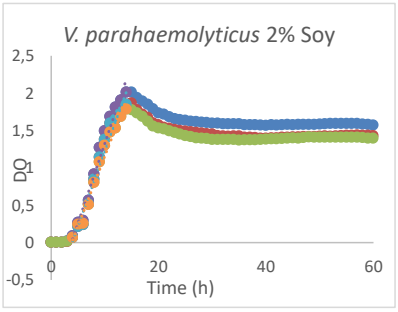


Figure 6: Growth rate curves of non stressed V. parahaemolyticus cells at 1% (wt/vol) of both salt and peptones.Four types of peptone: meat, casein, soy and yeast, were used.







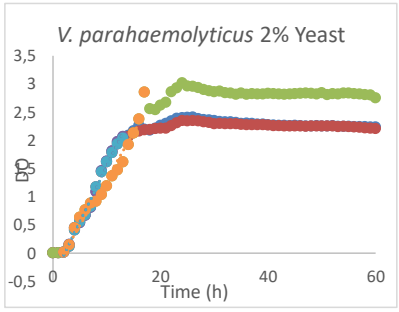
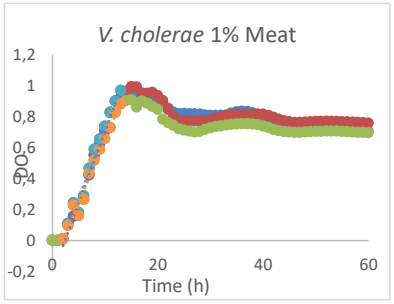
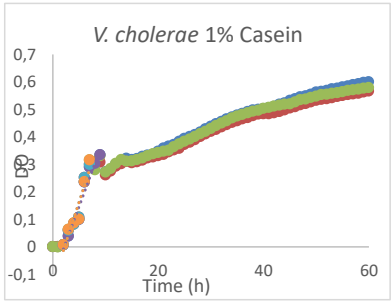
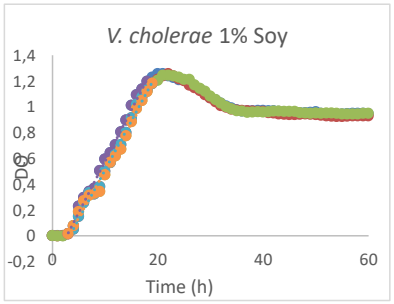


Figure 7:Growth rate curves of non stressed V. parahaemolyticus cells at 2% (wt/vol) of both meat and peptones.Four types of peptone: meat, casein, soy and yeast, were used.







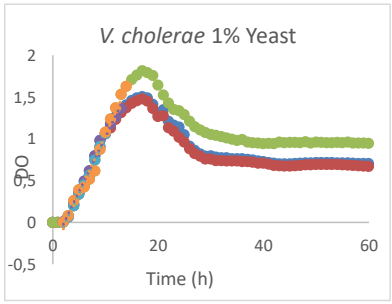


Figure 8: Growth rate curves of non stressed V. cholerae cells at 1% (wt/vol) of both salt and peptones. Four types of peptone: meat, casein, soy and yeast, were used.

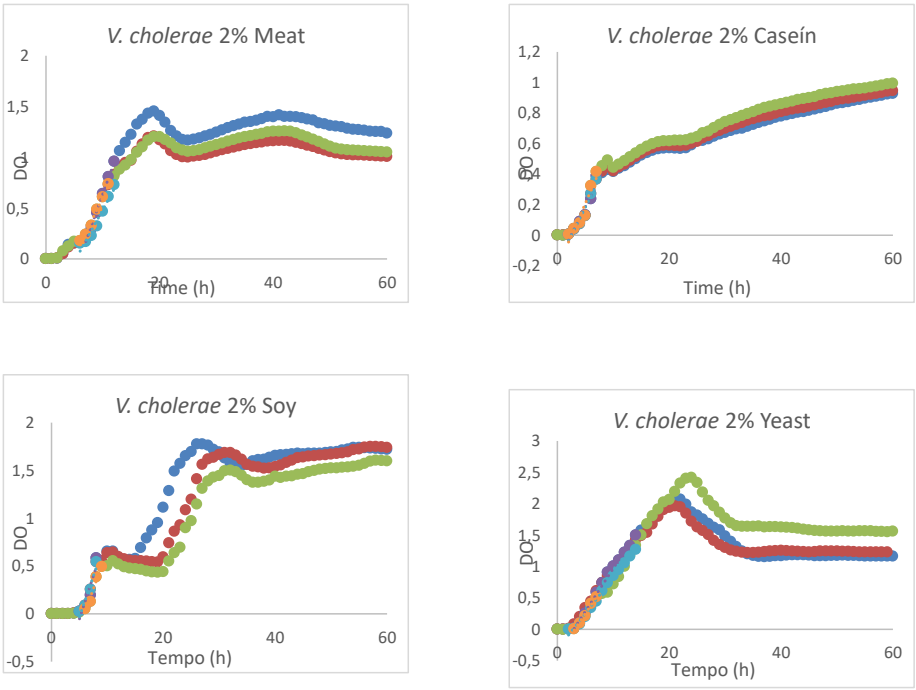


Figure 9: Growth rate curves of non stressed V. cholerae cells at 2% (wt/vol) of both salt and peptones . Four types of peptone: meat, casein, soy and yeast, were used.

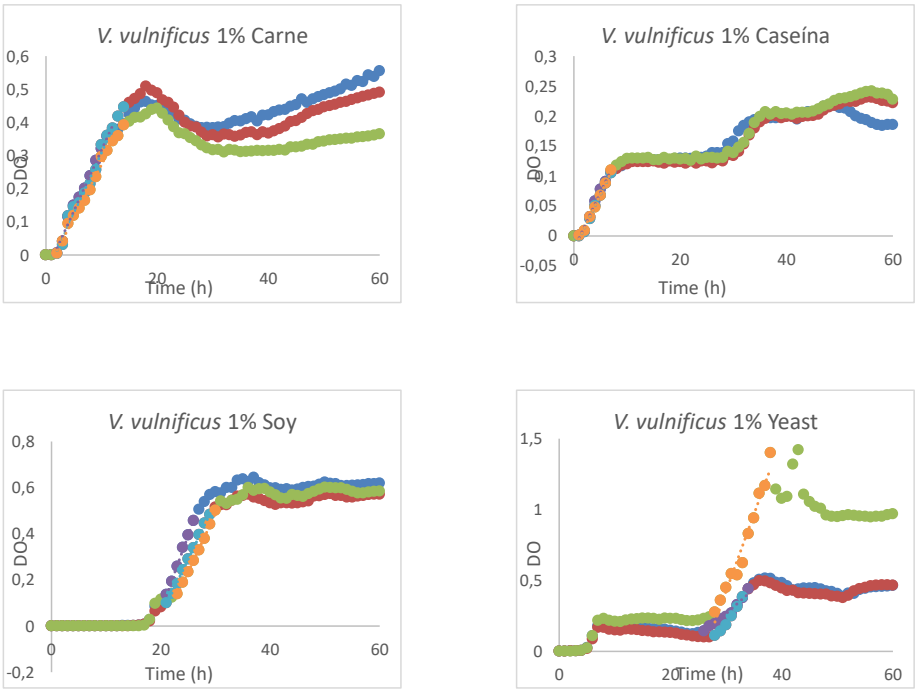
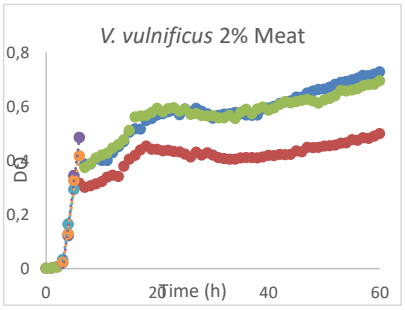
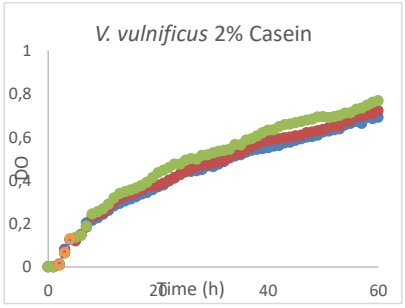


Figure 10: Growth rate curves of non stressed V. vulnificus cells at 1% (wt/vol) of both salt and peptones. Four types of peptone: meat, casein, soy and yeast, were used.





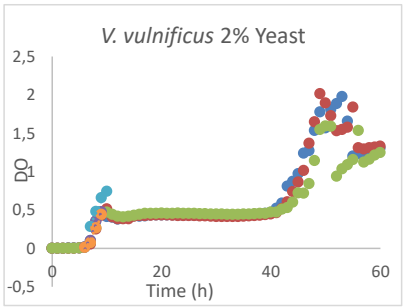


Figure 11:Growth rate curves of non stressed V. vulnificus cells at 2% (wt/vol) of both salt and peptones. Four types of peptone: meat, casein, soy and yeast, were used.

Lag time was measured as the number of hours without changing the OD value (0.0). Specific growth rate was calculated using the exponential points of the graphs. A straight line was fitted in those points and the slope was calculated as the specific growth rates for each condition (Zwietering, Jongenburger, Rombouts, Van ', & Riet, 1990).

ANNEX III – CALIBRATION CURVE

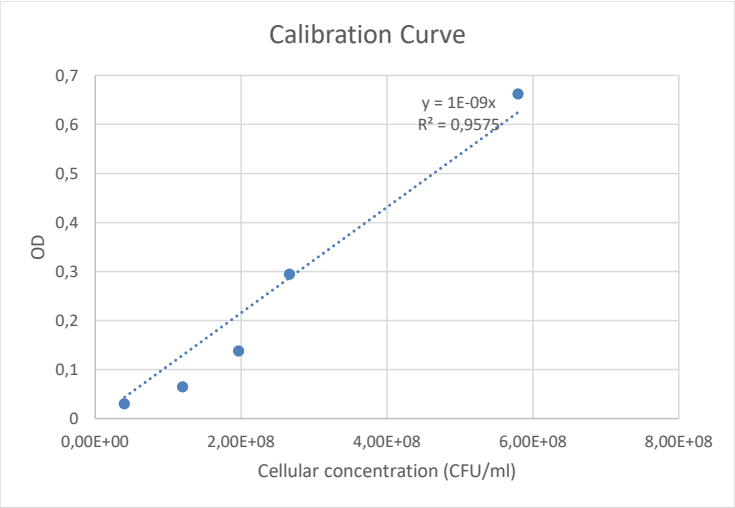


Figure 12: Correlation between the absorbance (optical density OD) and the cellular concentration for Vibrio cells. This experiment was performed with strain V. parahaemolyticus

ANNEX IV - MICROSCOPY VISUALIZATION RESULTS

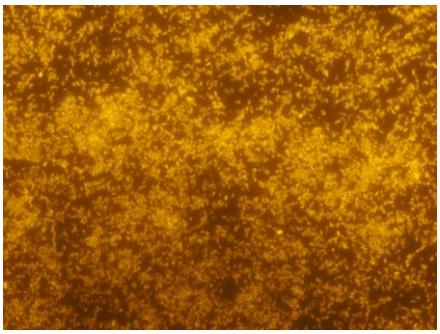


Figure 13: V. parahaeolyticus positive result.

Commented [CA1]: Faltam as escalas. Não precisam de ser muito exatas. Também podes usar o powerpoint para fazer isso.

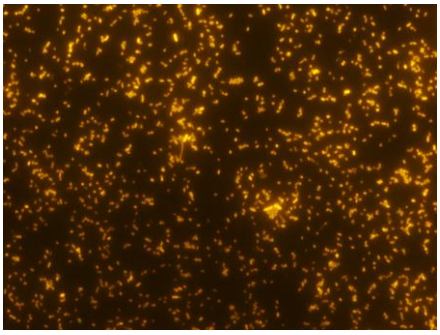


Figure 14: V. cholerae positive result.

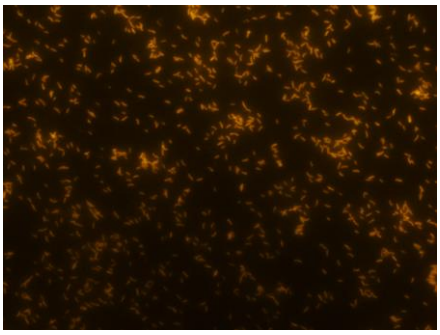


Figure 15: V. vulnificus positive result.