CENTRE ARMAND-FRAPPIER SANTÉ BIOTECHNOLOGIE

SELECTION OF APTAMERS AGAINST PATHOGENIC BACTERIA OF INTEREST IN AQUACULTURE AND PUBLIC HEALTH

By
Vanessa Aguilar Sánchez

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Evaluation jury

Jury president and internal examiner	Jean-François Naud INRS-IAFSB
External examiner	Maria C DeRosa Carleton University
Research director	Jonathan Perreault INRS-IAFSB
Research codirector	Sonia A. Soto Rodríguez CIAD

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ABSTRACT

In the present study, two enzymatic ssDNA production methods have been selected, and different combinations of them in order to study the possibility of improving the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) methodology, making faster cycles that can enrich the sequences in the same way.

In addition, several SELEX have been performed against ligands of pathogenic nature of interest in the food and aquaculture industry, as whole bacteria and a toxin complex causing AHPND disease (Acute hepatopancreatic necrosis disease) in shrimp farming.

An enzymatic method has been identified that allows the complete SELEX methodology in a few days, the use of digestion by lambda exonuclease enzyme has presented interesting results that show the selection of candidates with affinity for the ligand
In addition, some sequences have been selected that can be characterized as aptamers for ligands of interest and can be continued with the analysis of them under controlled conditions and directly in the field.

Keywords: SELEX, ssDNA, enzymatic digestion, aptamers.

RÉSUMÉ

Dans la présente étude, deux méthodes de production enzymatique d'ADN simple brin ont été sélectionnées, et différentes combinaisons des enzymes ont été essayées afin d'étudier la possibilité d'améliorer la méthodologie SELEX (Systematic Evolution of Ligands by Exponential Enrichment), rendant les cycles plus rapides que peuvent enrichir les séquences de la même manière qu'un SELEX traditionnel.

En plus, plusieurs SELEX ont été effectués contre des ligands de nature pathogène d'intérêt dans l'industrie alimentaire et l'aquaculture, comme des bactéries entières et une toxine formée par deux sous unités causant la maladie AHPND (Acute Hepatopancreatic Necrosis Disease) dans les cultures des crevettes.

Une méthode enzymatique permettant la complétion de la méthode SELEX en quelques jours a été identifiée. L'utilisation de la digestion par l'enzyme lambda exonuclease a présenté des résultats intéressants montrant la sélection de candidats avec une affiité pour le ligand.

Finalement, certaines séquences pouvant être caractérisées comme aptamères pour les ligands d'intérêts on été selectionées. Celles-ci ont été étudiées dans des conditions contrôlées.

Mots-clés : SELEX, sbADN, digestion enzymatique, aptamers.

SOMMAIRE RÉCAPITULATIF

Depuis 2003, on prévoyait une augmentation de 57% de la consommation de poisson dans les pays en développement et de 4% dans les pays développés pour 2020 (Halwart, 2009). L'aquaculture a produit, en 2016, 53% de la production totale de la pêche. Plus de 62 % de la valeur totale des produits de la mer et 17 % des protéines animales consommées dans le monde sont attribuables à l'aquaculture (FAO, 2018).

L'élevage de crevettes en Amérique Latine et dans les Caraïbes est une activité complexe, diversifiée et dynamique, elle pourrait facilement dépasser les 513 000 tonnes de production d'ici 2030. La concurrence croissante sur le marché continuera de faire baisser les prix et l'industrie est obligée de s'adapter pour améliorer sa compétitivité (Wurmann, Madrid, & Burgger, 2004).

Les virus et les bactéries sont responsables de la majorité des pertes associées aux maladies chez les crevettiers. Une pandémie virale et, plus récemment, une pandémie bactérienne ont mené à la conclusion que l'aquaculture durable de la crevette dépendra d'une plus grande efficacité. Le système actuel de production aquacole relativement ouvert dans les étangs extérieurs est sujet à des épidémies périodiques de maladies catastrophiques suivies de périodes de rétablissement de durée variable, en fonction des connaissances acquises de la recherche et de la capacité technique de chaque pays (Holmström, et al., 2003).

Une solution à titre préventif est l'utilisation d'un régime probiotique; les bactéries probiotiques ont le potentiel d'améliorer la culture de crevettes en réduisant les bactéries pathogènes et en améliorant la performance de croissance (Boonthai, Vuthiphandchai, & Nimrat, 2011), mais cela signifie que les agriculteurs doivent surveiller de près la santé animale afin d'identifier le stade de développement auquel le régime alimentaire est nécessaire, et ce service est normalement coûteux et avec des exigences matérielles spéciales.

Actuellement, les bactéries pathogènes sont préoccupantes dans le contexte de la santé mondiale; l'OMS estime que 600 millions de personnes dans le monde tombent malades chaque année en raison de la présence de ces bactéries, uniquement dans le secteur alimentaire.

Le risque d'infections par les aliments contaminés représente un risque élevé d'insécurité alimentaire. Le fardeau de ces maladies d'origine alimentaire est considérable, subies chaque année par environ 1 personne sur 10 et cause la perte de 33 millions d'années de vie saine (OMS, Organisation Mondiale de la Santé, 2018).

Certains des genres les plus courants de bactéries pathogènes qui représentent un grave problème dans la production alimentaire sont:

- *Escherichia coli*
- *Listeria monocytogenes*
- *Campylobacter jejuni*
- *Salmonella*
- *Yersinia enterocolitica*
- *Vibrio*

Selon l'OMS, les maladies infectieuses ont causé 32% des décès dans le monde, 68% en Afrique et 37% en Asie du Sud-Est. Ces maladies représentent 90 % des problèmes de santé dans le monde et tuent environ 14 millions de personnes chaque année, dont 90% dans les pays en développement.

DÉTECTION

La grande majorité des méthodes de diagnostic sont réalisées dans des laboratoires spécialisés, ce qui signifie qu'elles ne sont pas vraiment disponibles pour les producteurs sur le terrain. En particulier la classification des méthodes moléculaires; ces méthodologies fournissent un résultat diagnostique confirmatif et précis, mais qui, néanmoins, sont limités à des espaces, des ressources matérielles et humaines hautement spécialisés.

Les méthodes de diagnostic actuellement utilisées dans la plupart des exploitations agricoles des pays en développement sont les examens d'approximation, les examens microscopiques directs, la microbiologie classique et, dans quelques cas, l'histopathologie.

Ce facteur de disponibilité limite la capacité d'intervention sur le terrain en cas d'éclosions infectieuses qui menacent un grand pourcentage de la culture et, en même temps, engendre un besoin important de développer de nouvelles techniques de détection, qui nécessitent des ressources moins strictes et peuvent fournir une réponse rapide.

Un diagnostic rapide a non seulement un lien direct avec l'industrie et ses effets économiques, mais il est également de la plus haute importance dans la santé publique puisqu'un grand pourcentage des épidémies de maladies gastro-intestinales ont été liées à des aliments d'origine marine. Tel est le cas de l'intoxication alimentaire que provoque *Vibrio parahaemolyticus*. Cette bactérie est la cause de gastro-entérite associée à la consommation de produits marins contaminés. Une importante épidémie d'intoxication causée par cette bactérie a été identifiée au

Japon en 1953, où 272 personnes ont été touchées et 20 ont perdu la vie en raison de la consommation de sardine brute contaminée par *Vibrio parahaemolyticus*.

APTAMÈRES

Les aptamères sont des acides nucléiques simple brin qui ont une affinité pour un ligand spécifique. Leur capacité est fournie par des interactions intermoléculaires réalisées dans leur structure. Leur gamme de cibles est de très petites molécules organiques aux protéines et même aux cellules entières.

L'impact de l'utilisation des aptamères est présent dans des différents domaines d'étude, non seulement en tant que détecteurs de ligands d'intérêt mais aussi en tant qu'agents thérapeutiques pour différentes maladies.

PROBLÉMATIQUE

Les méthodes actuelles de détection des bactéries dans le domaine de l'industrie alimentaire et de l'aquaculture limitent le temps d'action, car la plupart d'entre elles nécessitent une manipulation et de longues périodes d'incubation. La présence d'agents pathogènes dans les produits de consommation humaine exige une méthode d'identification rapide et fiable afin d'avoir un meilleur contrôle et la possibilité d'un plan d'intervention immédiat.

HYPOTHÈSE

Les bactéries pathogènes présentes dans les échantillons d'intérêt de l'industrie alimentaire et de l'aquaculture peuvent être rapidement détectées à l'aide d'aptamères.

OBJECTIFS

- Sélection d'aptamères pour identifier les bactéries pathogènes d'intérêt
- Améliorer la génération de l'ADN simple brin par action enzymatique
- Sélectionner des séquences spécifiques pour la détection des ligands d'intérêt

MÉTHODOLOGIE ET RÉSULTATS

Deux méthodologies (lambda exonucléase et endonucléases) pour la production de sbADN ont été testées; l'action enzymatique a été utilisée pour digérer dbADN.

L'utilisation de l'exonucléase lambda pour la production de sbADN pendant le SELEX peut accélérer la méthodologie; le SELEX fait avec ce type de digestion enzymatique a été effectué en 6 jours, complétant un total de 14 cycles. Selon le temps d'incubation, il est possible d'exécuter la méthodologie complète en seulement quelques jours ouvrables, même lors de longues périodes d'incubation.

L'utilisation de la digestion nickase a causé des complications au cours de la méthodologie du cycle numéro 7, ce qui nous montre une possible interférence des petits fragments digérés en localisant rapidement leur partie complémentaire et en rejoignant du sbADN. L'interaction entre les candidats et le ligand est affectée par le fait de ne pas pouvoir prendre la structure tridimensionnelle qui donne à l'aptamère la possibilité d'identifier le ligand.

Les candidats contre *Vibrio parahaemolyticus* ont été sélectionnés par une combinaison de différents types de digestion enzymatique. Certains méthodes de production de sbADN sélectionées ont présenté des problèmes qui ont éventuellement affecté le bon développement et l'enrichissement des candidats. La caractérisation préliminaire de ceux-ci montre un pourcentage d'affinité qui n'est pas assez clair pour définir les séquences comme de vrais aptamères. En outre, au cours des SELEX suivants avec différents ligands, une des séquences caractérisées a été trouvée comme pollution dans les échantillons de ces autres mentionnés.

Les séquences sélectionnées pour *Campylobacter* et *Salmonella* montrent une présence répétitive dans le séquençage effectué, avec laquelle nous pouvons les définir comme séquences candidates.

Les séquences sélectionnées et caractérisées pour le complexe PirAB montrent une réponse d'identification intéressante, ainsi qu'un éventuel travail en équipe qui nous donne l'occasion d'élargir le type de biosenseurs ou de méthodologies qui pourraient être réalisés pour l'identification du ligand sur le terrain.

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ABREVIATIONS LIST

AHPND
Acute hepatopancreatic necrosis disease ..... v, vi, 24, 25, 26, 28, 48
APS
Ammonium persulfate 47
BFNNV
Barfin flounder nervous necrosis virus 40
BSA
Bovine serum albumin 45, 64, 86
CP
Coat protein 39, 41
D. F.
Dilution factor 50
DHPLC
Denaturing high-performance liquid chromatography 37
DNA
Deoxyribonucleic acid 32, 34, 36, 37, 40, 43, 44, 45, 46, 53, 57, 58, 59, 60, 64, 75, 77
dNTP
Deoxynucleotide triphosphates 45
dsDNA
Double stranded DNA 37, 46
EDTA
Ethylenediaminetetraacetic acid 47
ELISA
Enzyme Linked Immuno-Sorbent Assay 32
EMS
Early mortality syndrome 24, 25, 28
FP
Forward primer 58
FW
Forward primer 44, 45
GCRV
Grass carp reovirus 39

1 INTRODUCTION

1.1 Aquaculture and its development in Latin America.

Since 2003, there were forecasts of an increase of up to 57% in the consumption of fish of the developing countries, and 4% in the developed countries for 2020 (Halwart, 2009). Aquaculture produced, in 2016, a 53% of the total production of fishery. More than 62% of the total value of seafood and 17% of the animal protein consumed worldwide was attributed to aquaculture (FAO, 2018).

The commercial activity related to aquaculture in Latin America and the Caribbean is very significant, generating more than 200 000 jobs in 31 of the 44 countries of the continent. The production is made by cages, earthen, ponds, plastic, cement ponds (and others) of which Chile has 90% and where the most cultivated species are salmon and tilapia. In this country, there has been significant growth in salmon farming over the past two decades, where closed recirculation systems have been introduced to minimize the effects of aquaculture on the environment (Rojas A., 2009).

Shrimp farming in Latin America and the Caribbean is a complex, diverse and dynamic activity, reaching in 2017 a production of 2.9 million tons, valued at 17,400 million dollars in 2018 (Wurmann C. , 2019). Growing market competition will continue to press prices down and industry is forced into an adjustment to improve competitiveness. Here, development strategies include actions by governments and producer associations, promotional and marketing campaigns and the application of good management practices across the production and distribution chains. Some conflicts that aquaculture must cope with include the use of fishmeal, the introduction of exotic species and the water effluents are included in the first conflicts category, while objection to flooding some areas with salted water, the destruction of mangrove, sediments, wild animals, antibiotic residuals on the flesh, etc. are related to the second conflicts category. Occasional exports of shrimp, originating from certain areas of Asia, have been found to contain chloramphenicol residuals, or be contaminated with *Salmonella*, suggesting that prohibited medicines are being used to treat shrimp diseases, or that unsafe human handling has taken place after harvesting. Both situations can easily be avoided through adequate management practices (Wurmann, et al., 2004).

1.2 System intensity levels in aquaculture

We can define extensive, semi-intensive and intensive aquaculture in terms of production functions; as widely identified in the literature, the most extensive systems are those where there is little or even no human interference. As a consequence, those systems generally produce less than those of more intensity. A common type of an extensive system is where a restricted zone created by a net, cage or some type of a fence is inserted in a larger water body where animals can be cultured inside. Defining what distinguishes semi-intensive systems from extensive or intensive systems is not an easy task. Definitions of semi-intensive systems vary between countries and they do not always consider the same criteria. Lekang (2013) described semi-intensive system as a combination of an extensive and an intensive production and mentioned as an example an intensive fry production that is combined with an extensive on-growing rearing area. Semi-intensive systems have also been connected to feed and fertilization dependency. Describing intensive aquaculture systems, the input functions of supplying water, stocking and feeding are always applied while fertilizing and providing light are presented as optional. Intensive systems maintain high stocking levels and high feeding rates to maximize the production (Oddsson, 2020).

The use of different aquaculture systems in Latin American countries is variable, Hernandez-Rodriguez, et al., (2001) mention a summary of the systems used in some Latin American countries; Ecuador works 40% in an extensive system and the remaining 60% in a semi-intensive one. Mexico has the highest percentage of production (60%) through a semi-intensive system, 30% for the extensive system and 5% for the intensive system. Colombia has a total production through the semi-intensive system. Honduras with 20% extensive system and 80% semi-intensive system. Panama with 90% semi-intensive system and the rest with extensive system. Peru with 90% semi-intensive system and only 5% extensive system. Venezuela with its 100% semi-intensive system. All of them with a production of Penaeus vannamei as the most common species currently cultivated.

1.3 Shrimp diseases

Viruses and bacteria account for the majority of infection associated losses for shrimp farmers. Viral pandemic and, more recently, bacterial pandemic have led to the conclusion that future, sustainable shrimp aquaculture will depend on becoming of more efficient. When protective

measures fail and diseases occur in production ponds, there are currently only a few approved and practical therapeutic methods available for use with bacterial pathogens and none so far for viral pathogens. The current relatively open aquaculture production system in outdoor ponds is subject to periodic, catastrophic disease epidemics followed by recovery periods taking variable lengths of time, depending on knowledge gained from research and on the technical capability of each country. The **Figure 1-1** shows a record of Thai shrimp world production showing the cyclical nature of decreases because of disease epidemics followed by recovery after solutions were found (Flegel T. W., 2019). Some solutions can unleash certain problems like the commonly used antimicrobials; they may cause development of antibiotic resistance among pathogens infecting cultured animals and humans (Holmström, et al., 2003).

The evolution of resistance among microorganisms developed in two stages; in the first, bacteria evolved under different natural selective pressures and the final result was a characteristic phenotype of intrinsic resistance for any species of bacteria. The second era comprises the last decades since antibiotics began to be used in concentrations much higher than those found in natural environments, and is characterized by the accelerated evolution towards resistance of bacteria that were previously susceptible to antibiotics (acquired resistance) (Lara, et al, 2019).

Veterinary drugs are approved primarily for animals or poultry; however, they are also administered to farmed fish such as aquatic animals. These drugs are administered through feed and some by immersion. However, fish do not metabolize antibiotics efficiently and most of the administered dose is excreted (Salas, et al, 2021). These drugs enter the aquatic environment associated to organic material such as un-eaten medicated pellets and feces, and as a water-soluble fraction if eliminated via gills, feces and urine. It has been estimated that approximately 80% of the antimicrobials used in aquaculture enter the environment with their activity mostly intact (Love, et al, 2020).

Other possible preventive actions include the use of a probiotic diet. Probiotic bacteria have the potential to enhance shrimp culture by reducing pathogenic bacteria and improving growth performance (Boonthai, et al, 2011), but it means that the farmers need to closely monitor animal health in order to identify the stage of development at which the diet is required, and this service is normally expensive and has special material requirements.

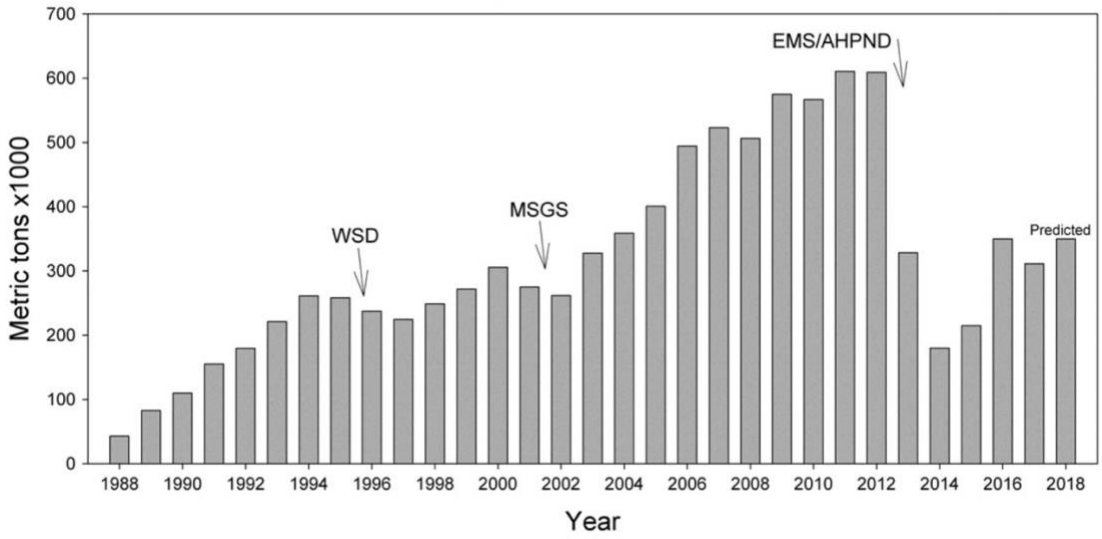


Figure 1-1 Cyclical nature of decreases because of disease epidemics

AHPND: acute hepatopancreatic necrosis disease; EMS: early mortality syndrome; MSGS: monodon slow growth syndrome; WSD: white spot disease. Taken from (Flegel T. W., 2019)**.**

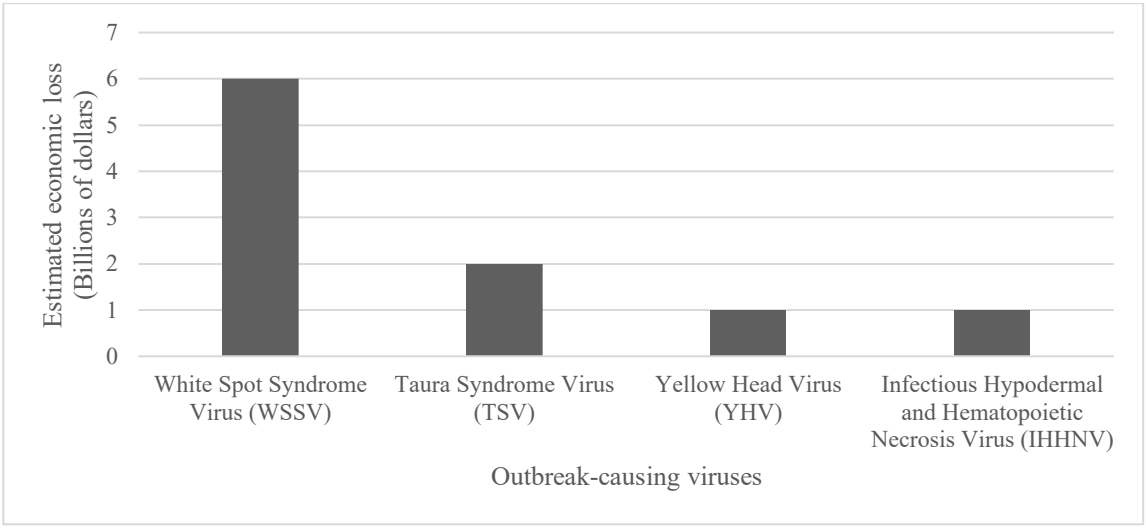
Aquaculture has had an important role as a pioneer within the food processing industry, as throughout history it has dealt with problems of conservation and disposal of a seasonally harvested perishable product (Delgado, 2003).

Aquaculture health plays an essential role in aquaculture as it provides a certain guarantee of quality in the final product, in addition to the fact that a timely detection of pathogens that infect crops has a direct effect on the percentage of losses on farms. For this reason, the development of diagnostic tools that can combine high sensitivity and specificity is particularly necessary to prevent, as far as possible, crop outbreaks (Bonami & Ronan, 2016).

Emerging infectious diseases have caused, over the last decades, a significant drop in productivity in countries with a high market share in aquaculture products. A clear example of this is the considerable decrease in production in China, Thailand, Indonesia and Mexico in 2013 due to the outbreak of the early mortality syndrome (EMS) (Dhar, et al., 2019); this animal health crisis caused a setback in aquaculture development in these countries, since a large number of producers were directly affected, thus reflecting the economic aspect of the activity. The Asian continent also presented serious percentages of mortality in the crops, this during the outbreaks of generalized diseases between 2012 and 2015. The great participation that Asia has in the aquaculture market results in the proliferation of outbreaks of emerging diseases, not only in the local market, since globalization allows the export of the disease quickly to the other continents.

This in turn generates serious problems throughout the production chain. The losses generated by these viral disease outbreaks, for example in shrimp production, have been catastrophic; valued in billions of US dollars (Dhar, et al, 2019). **Figure 1-2** shows, in a general way, the economic impact of some viruses that cause the main infectious diseases in shrimp in Latin America and the economic impact by AHPND around the world.

a)



b)

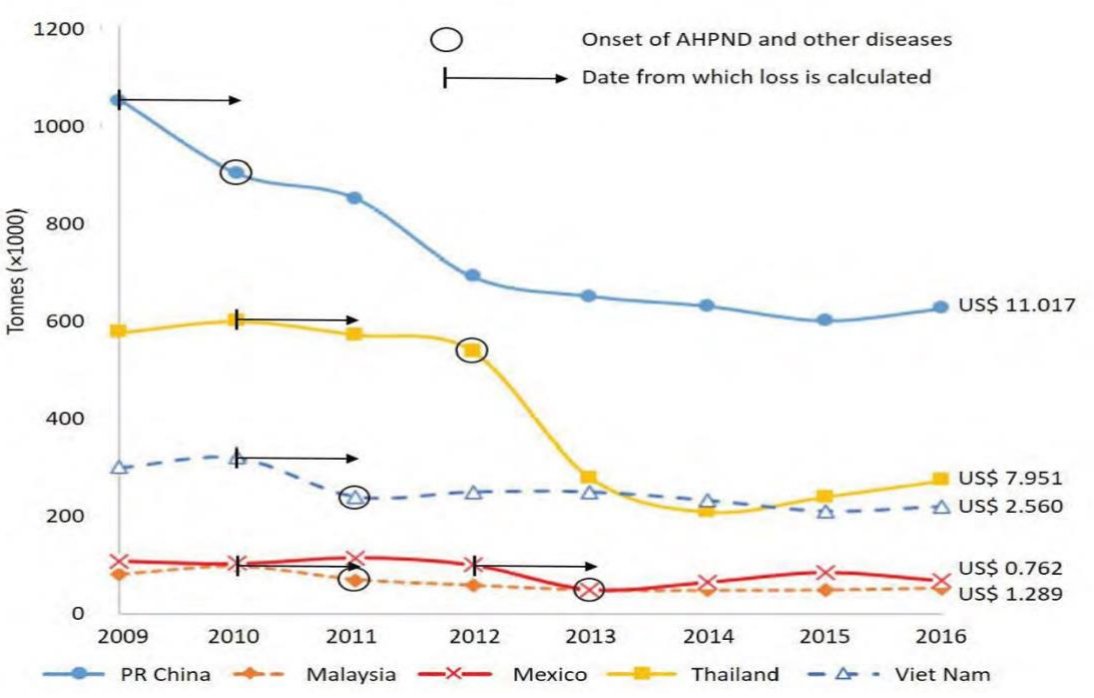


Figure 1-2 Economic impact caused by shrimp viral diseases and AHPND

a) Economic impact by viral disease in Latin America (2010-2018). Adapted from (Dhar, Cruz-Flores, Caro, Siewiora, & Jory, 2019)

b) Economic impact by AHPND. The first date of reported losses attributable to AHPND for each country is marked as a black circle, while losses are calculated from the black arrow. From (Shinn, et al., 2018).

1.3.1 The 2013 aquaculture crisis due to the Early Mortality Syndrome (EMS)

From 2008 to 2013, the cultivation of *Penaeus vannamei* in America represented 80.7% of world aquaculture production (Peña-Navarro, et al., 2013). This increase in the participation of the world market caused, in turn, a significant increase in the density of organisms cultivated by ponds, therefore, productive farms began to have a reduction in their water quality. This factor was of utmost importance since this characteristic gave pathogenic microorganisms the opportunity to develop more easily and freely in the aquaculture environment, thus generating an increase in the occurrence of opportunistic infectious diseases (Peña-Navarro, et al., 2013).

Due to the rise of aquaculture in the world market, emerging diseases advanced much faster, such as the early mortality syndrome (EMS) outbreak, later called the acute hepatopancreas necrosis disease (AHPND), which was first reported in 2009 in juvenile shrimps on farms in China, spreading from there to nearby countries such as Vietnam in 2010, Malaysia in 2011, Thailand in 2012 (Varela-Mejías & Peña-Navarro, 2014), the northwest of Mexico in 2013 and other countries (Soto-Rodriguez, et al., 2018).

The causal agent of AHPND was established in 2013 thanks to tests carried out at the Laboratory of Aquaculture Pathologies of the University of Arizona in the United States. It was found to be bacterial and related to *Vibrio parahaemolyticus*; which can be a carrier of a plasmid that contains a binary toxin that gives it pathogenicity. The clinical signs associated with the affected animals do not have a confirmatory diagnostic value, such as; erratic swimming, anorexia (Varelas-Mejía, et al., 2017) and expanded chromatophores, empty gut, pale and atrophied hepatopancreas (Soto-Rodriguez, et al., 2015).

Timely diagnosis can help to reduce high mortalities in shrimp culture, there are currently no preventive or therapeutic methods for the diagnosis of the toxins produced by *Vibrio parahaemolyticus* that cause AHPND. AHPND of *P. vannamei* has had a devastating impact on shrimp production and is now reported for at least eight Asian territories, and from Costa Rica, Honduras and Mexico (Shinn, et al., 2018).

1.4 Food-borne transmission diseases

Pathogens that affect the food industry and food supply security are not limited to those that cause diseases in livestock, poultry or aquaculture, human pathogens that spread through food are

probably an even greater concern. Currently, WHO estimates that 600 million people around the world get sick each year from the presence of these bacteria, only in the food sector.

The risk of infections from contaminated food represents a high risk of food insecurity. Among the most important bacteria, recognized as a causal agent of infections in the population, we find the genera *Salmonella* and *Campylobacter*, these genera are part of the four main causes of diarrheal diseases. Although infections by these pathogens can be controlled in most cases, in the case of children under 5 years old, the elderly and immunosuppressed individuals, they can be fatal. The burden of these foodborne diseases is considerable, suffered each year by about 1 in 10 individuals and causes the loss of 33 million years of healthy life (WHO, World Health Organization, 2018). There is a frequency of 550 million cases annually, of which 220 million are children under 5 years old. Furthermore, antimicrobial resistance is a global public health problem caused by these types of infectious agents; Salmonella is one of the microorganisms among which antimicrobial resistant serotypes that affect the food chain have appeared (WHO, World Health Organization, 2018). These genera are the most isolated agents in diarrhea of bacterial origin, both in industrialized countries and in those under development. Among the patients who become hospitalized, 62% refer to children under 2 years old (Turco, et al., 2014).

1.5 Pathogenic bacteria

There is a long list of cases where pathogenic bacteria are a source of contamination of food already present on the market, and it varies between the type of products such as frozen chicken, different types of lettuce, various beef products, seafood and others (Agency, 2020). Some of the most common pathogenic bacteria that represent a serious problem in food production are:

- *Escherichia coli*
- *Listeria monocytogenes*
- *Campylobacter jejuni*
- *Salmonella* spp
- *Yersinia enterocolitica*
- *Vibrio* spp

All these genera cause health problems that are aggravated in relation to the particular conditions of the host, the most affected being people with a compromised immune system. However, the importance of controlling these pathogens does not lie solely in the interest of public health, since

it also generates a high cost in the food production chain. In order to prevent the contamination of this type of microorganisms, it is not only enough to keep the surfaces clean, the machinery and even the material used during the disinfection of the equipment represent potential sources of contamination. In order to properly eliminate this risk in the food industry, we must first study the level of bacterial contamination of each surface in order to choose the best disinfectant to apply, without compromising the safety of the product. Nevertheless, there is a risk of producing food with traces of these disinfectants and affecting the consumer.

Food safety, nutrition and food security are inextricably linked. Unhealthy food creates a vicious cycle of disease and malnutrition, particularly affecting infants, children, the elderly and the sick (WHO, 2020).

1.5.1 Vibrio parahaemolyticus

Vibrio parahaemolyticus is a human pathogen that occurs naturally in marine environments and is frequently isolated from a variety of seafoods including codfish, sardine, mackerel, flounder, clam, octopus, shrimp, crab, lobster, crawfish, scallop and oyster. Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever. This bacterium is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States and an important seafood-borne pathogen throughout the world (Sua & Liub, 2007).

PirAB toxin.

Acute hepatopancreatic necrosis disease (AHPND) is a newly emerging shrimp disease caused by PirAB toxins encoded by a plasmid found in *Vibrio parahaemolyticus*. The PirAB toxins are the homologs of the *Photorhabdus* insect-related (Pir) toxins (Xiao, et al., 2017). AHPND, originally known as early mortality syndrome (EMS), is an emerging disease in shrimp. Since its first outbreak in 2009, the disease has caused serious global economic losses in the shrimp farming industry. It has been confirmed that *Vibrio parahaemolyticus* strains, which contain a 70-kbp plasmid, are the etiological agent of AHPND (Lee, et al., 2015). More specifically, the homologues of the *Photorhabdus* insect-related (Pir) toxins encoded by this plasmid are directly responsible for shrimp mortality in AHPND (Xiao, et al., 2017; Soto-Rodriguez, et al., 2015; Joshi, et al., 2014). The binary PirAB toxin is responsible for the characteristic lesions in the shrimp hepatopancreas (Soto-Rodriguez, et al., 2015). AHPND lesions have 3 phases: initial phase, there is an elongation of epithelial cells of the hepatopancreatic tubules and a reduction of vacuoles in B (produce and recycle fat emulsifiers and support digestion of material inside the tubular lumen) and R (support absorption and metabolization of nutrients, storage of energy reserves and minerals) cells; the acute phase, a distinctive feature of AHPND, is characterized by massive de tachment of the cells from the tubular epithelium into the lumen and dysfunction in embryonic (E) cells (stem cells); finally, the terminal phase is distinguished by the presence of an inflammatory response in the hepatopancreas (G3 [moderate, locally extensive to multifocal lesions] to G4 [severe, multifocal to diffuse damage] hemocytic infiltration accompanied by melanization of necrotic material) and proliferation of bacterial masses within the tubular lumen (Aguilar-Rendón, et al., 2020).

1.5.2 Salmonella enterica

Salmonella enterica is a zoonotic pathogen of substantial concern to global human and animal health; the most common foodborne pathogen isolated from food-producing animals (Jajere, 2019). It is a leading cause of morbidity and mortality in people worldwide. *S. enterica* can successfully colonize animals, humans, and plants, and is also found in the environment. Following ingestion, *S. enterica* invades the intestinal epithelium in the ileum and colon, either to cause a neutrophilic gastroenteritis or disseminate to systemic sites and cause sepsis. It thrives in the intracellular niche, allowing intrinsic antimicrobial resistance and chronic colonization in rare cases. *Salmonella* is transmitted by ingestion of contaminated food or water or by direct contact with infected individuals or animals (Knodler & Elfenbein, 2019).

The host specificity of particular pathogenic *Salmonella* depends on the serovar's ability to adapt to the environment of its hosts. This specific ability to adapt to the host's environment is regulated by many microbial characteristics, which are responsible for the expression of clinical manifestations in specific host species (Jajere, 2019). Enteric salmonellosis is usually self-limiting and is characterized by fever, abdominal pain, vomiting, and diarrhea. Children under the age of 5 years, the elderly, and immunosuppressed adults are at risk of systemic dissemination of the pathogen and require antimicrobial therapy to treat the infection. Symptoms of typhoid fever are fever, headache, lethargy, and anorexia, with only one-third of individuals experiencing intestinal symptoms. Antimicrobial therapy is required for successful resolution of infection (Knodler & Elfenbein, 2019).

1.5.3 Campylobacter jejuni

Campylobacter is one of the four main causes of gastroenteritis worldwide and the incidence and prevalence has increased in both developed and developing countries over the last decades (Cervantes-García, 2020). The genus *Campylobacter* is composed of 32 species, of which two are not yet well classified with nine subspecies (Cervantes-García, 2020). They occur primarily as commensals in humans and domestic animals. The thermophilic campylobacters *C. jejuni, C. coli, C. lardis* and *C. upsaliensis* are more associated with human gastrointestinal disease, especially *C. coli* and *C. jejuni ssp. Jejuni* which account for 95% of all clinical isolates in the UK. *C. jejuni*, the most common species of *Campylobacter* associated with human illness is prominently associated with poultry and has evolved to preferentially colonize the chicken gut given its optimal growth conditions, e.g. 42°C (Snelling, et al., 2005).

1.6 Economic impact of infection by pathogenic bacteria

Infectious diseases constitute a tenacious and major public-health problem all over the world. Although some, such as smallpox and poliomyelitis, have been eradicated from nature or almost wiped out, many diseases persist with little hope of getting them under control. In addition, new infectious diseases are emerging and old ones that were thought to be under control are regaining lost ground. According to the US National Institutes of Health, 16 new infectious diseases have been identified in the past two decades; five others have been identified as re-emerging (Fonkwo, 2008). Emerging infections can be defined as infections that have newly appeared in the population or that existed previously but are rapidly increasing in incidence or geographic range (D. M. Morens & Fauci, 2004)

Moreover, during the past couple of decades microbes have shown a tenacious ability to adapt, re-adapt, survive and challenge human ingenuity. The impact of these diseases is immense and is felt across the world. In addition to affecting the health of individuals directly, infectious diseases are also having an impact on whole societies, economies and political systems. The infectious agents not only take an enormous physical toll on humanity, but also cause significant economic losses both directly in the developing world and less directly in the developed world. It is therefore a matter not only of public health, but also of economic interest, to invest in and organize an internationally coordinated strategy to fight the major infectious diseases, or at least to bring them under control (Fonkwo, 2008).

The 2019 World Bank report on the economic burden of the foodborne diseases indicated that the total productivity loss associated with foodborne disease in low- and middle-income countries was estimated at US$ 95.2 billion per year, and the annual cost of treating foodborne illnesses is estimated at US$ 15 billion (WHO, World Health Organization, 2020).

The 2015 WHO report on the estimates of the global burden of foodborne diseases presented the first-ever estimates of disease burden caused by 31 foodborne agents (bacteria, viruses, parasites, toxins and chemicals) at global and sub-regional level, highlighting that more than 600 million cases of foodborne illnesses and 420 000 deaths could occur in a year. The burden of foodborne diseases falls disproportionately on groups in vulnerable situations and especially on children under 5, with the highest burden in low- and middle-income countries (WHO, World Health Organization, 2020).

The foodborne diseases account for 90% of the health problems worldwide and kill about 14 million people annually, 90% of whom are from the developing world (Fonkwo, 2008). Tuberculosis (TB), for example, is increasingly being acknowledged as an important factor in the political and economic destabilization of the developing world. Country studies document that each TB patient loses, on average, 3–4 months of work time annually due to the disease, and lost earnings amount to 20–30% of household income. Families of people who die from the disease lose approximately 15 years of income. The global burden of TB in economic terms can therefore be easily calculated: given 8.4 million patients yearly according to the most recent WHO estimates, the majority of whom are potential wage-earners, and assuming a 30% decline in average productivity, the toll amounts to approximately US$1 billion each year (Fonkwo, 2008).

US$110 billion is lost each year in productivity and medical expenses resulting from unsafe food in low- and middle-income countries. Foodborne diseases impede socioeconomic development by straining health care systems, and harming national economies, tourism and trade. Food supply chains now cross multiple national borders. Good collaboration between governments, producers and consumers helps ensure food safety. Faced with the growth of global industrialization, the difficulties caused in production by pathogenic microorganisms imply greater responsibility for producers. Local incidents can quickly evolve into international emergencies due to the speed and range of product distribution (WHO, World Health Organization, 2020).

1.7 Methods of detecting pathogenic bacteria in aquaculture industry

One of the ways to better control the impact of pathogenic microorganisms on the food industry is a fast way of detecting the pathogens. For a diagnosis of diseases in aquaculture it is necessary to define the type of sampling to be carried out, which can be random (when what is required is to define the health status or the prevalence of a pathogen), or not random (in situations where organisms clearly exhibit pathology signs). Once the sampling is defined, degrees of severity are assigned for the lesions or infectious process to be visualized (Lightner & Pantoja, 2003).

Within the different methods used we find two classifications: classical methods and molecular methods. Both groups described by Lightner & Pantoja in 2003 and by the Manual of diagnostic tests for aquatic animals in 2020 are detailed below.

1.7.1 Classical methods

• Approach exam; external appearance and clinical symptoms are examined in order to find macroscopic symptoms that can guide the type of subsequent analysis or the choice of individuals to sample.
• Direct examination under a microscope; fresh mount parts of the internal organs that present macroscopic symptoms are examined in order to identify the degrees of severity and define a phase of the disease. Electron microscopy is also used.
• Microbiology (isolation and culture); samples are taken from internal organs lesions, as well as hemolymph from the organisms to be analyzed, in addition to water and soil samples to be seeded in specific microbiological media for the identification of the suspected pathogen.

• Histology; histological analyzes are used to clearly identify the stage of infections. According to the characteristics of these lesions, it is possible to diagnose the stage of the disease, however, it is limited only to the diagnosis of individuals with visible lesions.

1.7.2 Molecular methods

• PCR; PCR methods that target Vp_{AHPND} toxin genes have been developed (Sirikharin, et al., 2014; Tiwongger, et al., 2014; Han, et al, 2015; Flegel & Lo, 2014). The AP3 method is a one-step PCR targeting the 12.7 kDa $PirA^{vp}$ gene (Sirikharin, et al., 2015). Soto-Rodriguez *et al.* 2015, reported that the AP3 method produced the highest positive (90%) and negative (100%) predictive values of the PCRs tested (OIE, 2020).

• Serological tests with immune serum; the type of sample required for this type of tests must contain antibodies to the antigens or genetic probes for nucleic acids of the pathogen that need to be detected, for which a homogenate of the entire organism or of the particular organ is used.

•Serological tests with monoclonal antibodies.

- Fluorescent antibodies; using antibodies attached to fluorescent molecules to observe their binding to the specific pathogen.
- ELISA (Enzyme Linked Immuno-Sorbent Assay); detection of the specific antigen.

• Genetic probes (with radioactive or non-radioactive locating agent).

- "Dot blot" hybridizations; detection by nucleotide or antibody probes.
- In situ type hybridizations (in histological sections); it allows the detection of genetic sequences, or another type, from the pairing of complementary DNA sequences.

The diagnostic methods that are currently used in most of the cultivation farms in developing countries are fresh preparations, direct microscopic examinations, classical microbiology and in a few cases histopathology. The vast majority of diagnostic methods are performed in specialized laboratories, this means that they are not truly available to field producers. Specially the classification of molecular methods; these are methodologies that provide a confirmatory and precise diagnostic result, but their availability is limited to highly specialized spaces, material and human resources. This availability factor limits the ability to respond in the field to infectious outbreaks that threaten large percentages of the crop. This generates a significant need to develop new detection techniques, which require less strict resources and can provide a fast response.

1.8 Public health importance

A timely diagnosis not only has a direct relationship with the industry and its economic effects, it is also of utmost importance in public health since a large percentage of outbreaks of gastrointestinal diseases have been related to foods of marine origin. In such cases, a timely detection and a short response time is not easy to apply in the field due to the characteristics of the work areas and the requirements of current detection techniques, a rapid "on-site" detection would allow a larger range of corrective actions and decrease the chances that the pathogen in question reaches the consumers causing infections and outbreaks.

A good example is food poisoning caused by *Vibrio parahaemolyticus*. This bacterium is the cause of gastroenteritis associated with the consumption of contaminated marine products. A major outbreak of poisoning caused by this bacterium was identified in Japan in 1953; where 272 people were affected and 20 lost their lives due to the consumption of raw sardine contaminated by *Vibrio parahaemolyticus*. Later, in 1996 a pandemic clone of *V. parahaemolyticus* serotype O3: K6 caused outbreaks in Taiwan for 3 years to expand in 1999 to Asia, Russia, Mozambique, the United States, Canada and Chile. In 2004 it arrived in Mexico with more than 1,250 cases, which were attributed to shrimp consumption. Shrimp farming in Mexico is in the first place of fish/seafood production, and its main export destinations are the United States, Japan and France (Rodríguez-Camacho, et al., 2014).

The relationship between pathogens present in aquaculture production and public health is not only limited to gastrointestinal disorders. *Vibrio vulnificus*, an aquatic bacterium that causes epizootics or outbreaks of hemorrhagic septicemia with high mortality rates in fish, is also capable of infecting humans and causing death from sepsis, generally after consuming raw shellfish, or infections in wounds exposed to fish or seawater, and is therefore classified as a zoonotic species (González, 2010).

1.9 Biosensors

Detection of pathogens has been key to control of outbreaks, both in food industry and for the population. However, as mentioned above, diagnosis/detection methods either lack precision or require specialized equipment and trained laboratory professionals. The use of biosensors allows to meet the objective of having an effective and rapid detection tool to avoid outbreaks and, with them, economic losses and deterioration in public health. The use of biosensors also allows to eliminate the specialized needs of currents techniques.

When a device uses a biomolecule for recognition it is called a biosensor. The use of aptamers ("receptor-like" single-stranded DNA or RNA oligonucleotides selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Tuerk & Gold, 1990)) as tools capable of generating specific analytical information in the presence or absence of a ligand, has been the subject of study in recent years. **Figure 1-3** shows the elements of a biosensor, necessary to send an "acknowledgment signal".

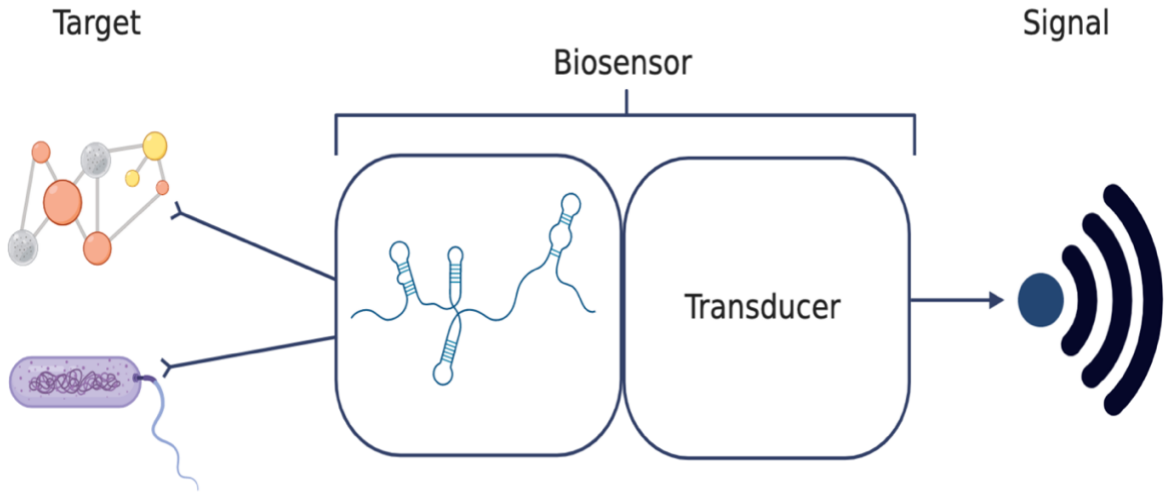


Figure 1-3 Biosensor elements with aptamers as reconnaissance element

Biosensors can be classified according to their recognition element into: enzymatic, whole cell or affinity. Affinity biosensors bind to specific targets or groups of them that have a relationship based on their structure. They are characterized by being very sensitive, selective and versatile. The use of antibodies as a recognition element gives biosensors these characteristics, and many other advantages such as the possibility of being transportable devices in the field, they can be fast and inexpensive (Ponce, 2015).

Among the advantages provided by the use of aptamers as a recognition element, we can mention the resolution of the ethical problem of the use of animals for experimentation, since the generation of natural antibodies requires in vivo procedures, in addition, it is really difficult to successfully obtain antibodies for small organic molecules which do not generate an immune response from the host.

1.10 Aptamer applications

Aptamers are one of the most studied types of “specific recognition element” used in biosensors. Aptamers are single-stranded DNA or RNA oligonucleotides selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) with binding ability to target biomolecules with high affinity and specificity (Tuerk & Gold, 1990). There are several advantages to the use of aptamers as compared to antibodies: aptamers bind target molecules with affinity and specificity equal or superior to those of antibodies, they are easily synthesized and modified, and they are more stable than antibodies. In light of these properties, studies on aptamers used as therapeutic, diagnostic and analytical reagents have become more important (Duan, et al., 2013). The application of aptamers as an emerging technology includes the diagnosis of diseases, therapies, detection of drugs and other substances such as contaminants, validation of targets, as well as molecular imaging. Biosensors have operational advantages such as rapid detection, portability, easy mass production and low cost for their application and use in the diagnosis of infectious diseases. However, the advantages of using aptamers are not only limited to these types of diseases, they also continue to be developed for the diagnosis of non-infectious diseases such as cancer or myocardial infarction (Chong & Low, 2019).

1.10.1 Aptamers for the detection of small organic molecules

One of the most severe problems facing environmental pollution is the impact it has due to the various economic activities that generate pollutants in their daily work, such is the case of agrarian activity. An important factor in this matter is the timely detection of polluting molecules, in order to reduce the risk of consequences both in public health and in the environment. In many developing countries, environmental monitoring is not sufficiently effective to generate a reliable data history, since the analysis techniques required for good monitoring imply complex tools and human resources with good technical training. Some of the techniques used are gas or liquid chromatography and mass spectrometry, which involves detection of electron capture. These techniques, in addition to counting the aforementioned conditions as a limitation, require prior treatment of the samples, which increases their cost and response time. Between 1998 and 1999, the Secretariat of Science and Technology of the Ministry of Culture and Education of the Argentine Nation, highlighted as an opportunity area the development of new analytical strategies for the analysis of complex mixtures oriented to soil analysis (Ponce, 2015).

1.11 SELEX

Systematic evolution of ligands by exponential enrichment (SELEX) is a well-established and efficient methodology for the generation of oligonucleotides with a high target affinity. It is an iterative in vitro process where the oligonucleotides are selected and amplified from a large library of random combinations. Each of these random chains take on a unique three-dimensional structure that gives them the ability to bind to a specific ligand. These SELEX-derived single stranded DNA and RNA molecules (aptamers) can be selected against various targets, such as proteins, cells, microorganisms, chemical compounds etc. They have a great potential for uses as antibody-like molecules, in cancer theragnostics and in biomedical research. Vast interest in aptamers stimulated continuous development of SELEX, which underwent numerous modifications since its first application in 1990. Novel modifications made the selection process more efficient, cost-effective and significantly less time-consuming. Most aptamers selected through SELEX (systematic evolution of ligands by exponential enrichment) exhibit high affinity to targets with a dissociation constant (Kd) in the micromolar to nanomolar range (Darmostuk, et al., 2015).

Aptamer molecules have gained much attention in the scientific field. This increasing attraction can be attributed to their many desirable traits, such as 1) their potentials to bind a wide range of molecules, 2) their malleability, due to its ease of modification and its stability and 3) their relatively low cost of production. These traits have made aptamer molecules an interesting platform to pursue in the realm of pharmaceuticals and biosensors. In essence, aptamer selection begins with the generation of a large diverse oligonucleotide library with predesigned primer-binding domains for PCR amplification. The library is introduced to a target of interest; and sequences demonstrating affinity for the target of interest are separated from those unbound sequences. Next, the bound oligonucleotides are collected and PCR amplified for subsequent rounds of enrichment. This process is repeated until the library converges onto a collection of sequences that demonstrate affinity of the target of interest. Finally, the final library is sequenced (Wua & Kwon, 2016).

1.11.1 Negative and positive selections

A significant challenge encountered when performing SELEX against complex target mixtures is deriving aptamers that are specific for the intended target. In conventional SELEX formats, a single purified protein provides the “bait” for recovery of target-specific aptamers. Various

techniques that allow for facile partitioning of aptamers from nonbinders may be used, including affinity beads, hydrophobic plates, or nitrocellulose filters. Counter-SELEX or “negative selection” may reduce the possibility of evolving nonspecific binders or improve the odds of recovering aptamers specific for one target over another. Counterselection techniques have been used efficiently with small molecules. Similar principles are applicable to complex targets, allowing identification of aptamers that bind to a specific cell surface target or to a specific cell population. In either case, counterselection strategies are critical given the multitude of potential “aptogenic” epitopes, that is, epitopes against which aptamers can be generated readily that are common to the surface of many cell types. The presentation of a complex target during SELEX without counterselection allows for the parallel selection of aptamers to multiple targets in a single selection. Aptamers that recognize multiple forms of a specific target can also be obtained by sequential target selection (Shamah, et al., 2008).

The negative followed by the positive selection steps are carried out to filter out sequences against the molecules existing on the surface of both the target and control cell lines. These steps should be repeated several times to enrich the aptamer pool for the target. (Chen, et al., 2016).

1.11.2 Generation of single stranded DNA (ssDNA)

Because perfectly double stranded DNA (dsDNA) does not adopt complex structures, ssDNA needs to be produced for each round of SELEX. There are several methods reported to generate ssDNA from double-stranded PCR products, including asymmetric PCR, denaturing high-performance liquid chromatography (DHPLC) method, lambda exonuclease digestion, size separation by denaturing urea–polyacrylamide gel derived from unequal primers with chemical modification and magnetic separation of biotin-labeled aptamers with streptavidin-coated beads (Chen, et al., 2016).

Lambda exonuclease is an enzyme involved in the repair of double-stranded breaks of the viral DNA. As an exodeoxyribonuclease, it selectively digests the phosphorylated strand(s) of double stranded DNA (dsDNA) from the 5′ to the 3′ end. Having 20 times more affinity for a phosphorylated 5′-end than a hydroxylated 5′-end, only non-phosphorylated single-stranded DNA remains after digestion (Marimuthu, et al., 2012). A fast and efficient method of generating ssDNA with high quality and yield relying on lambda exonuclease digestion is possible (Marimuthu, Tang,

Tominaga, Tanc, & Gopinath, 2012). It has a drawback that incomplete digestion may lead to the contamination of the dsDNA in a reaction mixture (Chen, et al., 2016).

Generation of ssDNA is not only important in SELEX, but also for other applications such as pyrosequencing, solid-phase DNA sequencing DNA single-strand conformation polymorphism analysis, nuclease S1 mapping, probe preparation and labelling, subtractive hybridization, as well as for DNA chips and microarray (Svobodová, et al., 2012).

1.11.3 SELEX against whole bacteria (cell-SELEX)

In addition to the most common aptamer targets, i.e. proteins, SELEX can also be performed to find aptamers to recognize particular types of cells. In cell-SELEX, aptamers are developed against molecules on the cell surface without requirement for prior knowledge of the molecular targets. Large transmembrane molecules are functionally important molecules involved in many biological processes, such as signal transduction, cell adhesion and migration, cell–cell interactions, and communication between the intra- and extra-cellular environments. Yet the membrane proteins aptamers developed through protein-based SELEX may not be able to selectively recognize and interact with their corresponding targets within the context of a cell, which would result in failure of the bio-medical application. In cell-SELEX, all molecules on the cell surface are in their native states and would therefore represent their natural folding structures and distribution. Therefore, cell-SELEX eliminates the risk that identified aptamers would only bind to the purified proteins but could not recognize the native form of the proteins on living cells (Chen, et al., 2016).
In summary, cell-SELEX has as a specific ligand only one of all molecules present on the cell surface, without this specificity being defined from the beginning, the aptamers are selected based on the enrichment that is achieved with the cycles and the ligand can be analyzed once the candidate has been obtained; during its characterization.

1.12 Apta-sensors and its advantages in aquaculture industry

Recent decades have witnessed the rapid development and wide application of aptamers in a variety of science fields, including pathogen detection, disease diagnosis, targeted therapy, pathogenesis research, and bioanalytical applications. Aptamers are considered to be “artificial antibodies.” Just like biological antibodies, aptamers recognize targets with

high specificity and affinity. Many aptamers have been developed against different targets, including proteins, purified virus particles, virus-infected cells, and diseased tissues. Dozens of specific aptamers for aquatic pathogenic bacteria have been reported (**Table 1-1)**, and they have great potential for rapid detection of pathogenic bacteria and water pollution (Yu, et al., 2021).

Table 1-1 Aptamers targeting aquatic pathogens and toxins

Name	ssDNA or RNA aptamers	Targets
A1P. A3P, A17P, A18P, A21P	ssDNA	*Vibrio parahaemolyticus*
Vapt2	ssDNA	*Vibrio vulnificus*
Apt-1, Apt-2, Apt-3. Apt-4	ssDNA	*Vibrio parahaemolyticus*
VA2, VA8	ssDNA	*Vibrio alginolyticus*
Family 1, 2, 3, 4, 5, 6, 7, 8, 9	ssDNA	*Vibrio alginolyticus*
C14, C22	ssDNA	*Vibrio harveyi* and *Vibrio parahaemolyticus*
A15	ssDNA	*Listeria monocytogenes*
S1	ssDNA	*Shigella dysenteriae*
ST2P	ssDNA	*Salmonella typhimurium*
S8-7	ssDNA	*Salmonella typhimurium*
S6	ssDNA	*Salmonella typhimurium*
Sp1, Sp20	ssDNA	*Shigella sonnei*
Se1, Se2, St1	ssDNA	*Salmonella enteritidis* and *S. typhimurium*
SAL 28, SAL 11, SAL 26	ssDNA	*Salmonella enterica* Serovar typhimurium
LMB-761, LMB-764, LMB-748, LMB-439, LMB-755, LMB-767	ssDNA	Singapore grouper iridovirus (SGIV)
Q2, Q3, Q4, Q5, LYGV1, LYGV2, LYGV3, LYGV4	ssDNA	SGIV-infected cells
QA-92, QA-9, QA-12, QA-36, QA-17, QA-79, QA-41, QA-88	ssDNA	Soft-shelled turtle iridovirus (STIV)
A5, A10, B11	ssDNA	Coat protein (CP) of redspotted grouper nervous necrosis virus (RGNNV)
TNA1, TNA4, TNA19, GBN2, GBN10, GBN34,	ssDNA	RGNNV-infected cells
H1, H2, H3, H4	RNA	Hirame rhabdovirus (HIRRV)
F1, F2, C6	RNA	Viral hemorrhagic septicemia virus (VHSV)
F2, F7, F10, F13, T10	ssDNA	S10 protein of grass carp reovirus (GCRV)
APT(STX1), M-30f	ssDNA	Saxitoxin (STX)
GO18-T-d	ssDNA	Gonyautoxin 1/4

Adapted from (Yu, et al., 2021).

1.12.1 RNA aptamers against viral hemorrhagic septicemia virus (VHSV)

Viral hemorrhagic septicemia ranks among the viruses of greatest concern at the industrial level for aquaculture, and not only for this area as it also threatens marine wildlife. This concern has generated the need to develop tools, both for detection and for therapeutic use, that give the industry and the animal health area the possibility of reacting in a timely manner to infection (Punnarak, et al., 2012). Punnarak et al. showed the results of their experiments in 2012, in which they demonstrated the ability of three aptamers (F1, F2 and C6), not only to bind VHSV but also to inhibit their growth (Punnarak, et al., 2012).

1.12.2 DNA aptamers specific binding to Singapore grouper iridovirus (SGIV)

Singapore grouper iridovirus (SGIV) it is a new virus belonging to the genus Ranavirus, family *Iridoviridae*; causing major economic losses in the aquaculture industry (Yu, et al., 2017). In the last decades SGIV positioned itself as the main viral pathogen causing great losses in China and Southeast Asia.

Li et al. reported in 2014 the development of a ssDNA aptamer capable of blocking SGIV infection in fish cells and in vivo cultures. The reported experiments showed two aptamers (LMB-761 and LMB-764) capable of reducing the relative mortality of SGIV without causing negative effects. *In vivo* experiments in the control group showed an accumulated mortality of 90% on day 7 post infection, while the group of organisms treated with the LMB-761 and LMB-764 aptamers had a mortality of 50% and 60% respectively (Li, et al., 2014)

A year later, in 2015, Li et al. again reported aptamers of specific recognition for SGIV in fish, this time in infected spleen cells. Showing four aptamers (Q2, Q3, Q4 and Q5) with good response in the experiments; particularly Q2 showed a high affinity for infected cells, with a binding affinity of 12.09 nM. The experiments were also carried out in vivo where Q2, Q3, Q4 and Q5 did not show cytotoxic effects on fish, even when the concentration of aptamers approached 1000 nM. One year later, Q3 was used to identify the presence of SGIV infection in samples of kidney, liver and spleen in vivo (Li, et al., 2016).

Aptamers have become popular in use as biosensors due to their low incidence of toxicity and their easy modification and manipulation. In fish, RNA aptamers have also been shown to provide

protection against the viral hemorrhagic septicemia virus (VHSV) and Hirame rhabdovirus (HIRRV) (Li, et al., 2014).

1.12.3 Aptamers versus virus in aquaculture

The group of nervous necrosis viruses (NNV) it is one of the most fatal viruses in aquaculture, with an infectivity of about 70 different species. It is capable of causing mortalities of up to 95% in cultivation farms. However, there are currently a low number of studies focused on the development of diagnostic or therapeutic techniques with the aim of reducing the effects of this type of virus in aquaculture. The most effective way to control infections caused by this pathogen is a combination of prevention and treatment actions. An extremely useful way to facilitate this goal is to develop rapid detection techniques (Yu, et al., 2019).

Yu et al. characterized three aptamers (TNA1, TNA4 and TNA19) capable of showing high specificity against *Trachinotus ovatus nervous necrosis viruses* (GTONNV), a virus isolated from infected organisms of the golden pompano species (Yu, et al., 2019). The selected aptamers also showed antiviral effects against GTONNV, both *in vitro* and *in vivo*. The antiviral activity of the aptamers was analyzed by qRT-PCR (Quantitative Reverse Transcription Polymerase Chain Reaction), the results showed that, relative to the control group, the relative expression of mRNA of the genes of the NNV coat protein (CP) and RNA-dependent RNA polymerase (RdRp) decreased significantly in infected cells treated with aptamer candidates. The *in vivo* analysis consisted of a control group of infected organisms, which showed a mortality of 40% on day 1 post-infection and a cumulative mortality of 90% on day 5. While in the infected organisms treated with the aptamer mortality started until day 3 post-infection and cumulative mortality peaked at 60% on day 9. Indicating *in vivo* protection by the aptamer against NNV infection (Yu, et al., 2019).

2 PROBLEM STATEMENT, HYPOTHESIS AND OBJECTIVES

2.1 PROBLEM STATEMENT

Current detection methods for bacteria in the field of food industry and aquaculture are limiting in terms of action time because most of them require handling and long incubation times, in addition to specialized equipment and experienced working staff. These requirements reduce the possibilities of the industry to implement corrective actions, thus presenting a significant increase in economic losses.

In addition, in the area of public health, the presence of contamination by pathogens in human consumption products requires a fast and reliable identification method to have better control and the possibility of an immediate response plan.

The use of biosensors, more specifically biosensors based on aptamers, could allow the solution of the problem of rapid and precise detection. However, on the way to obtaining the tools we found that a lot of time is typically required for ssDNA production. There are many methods for obtaining ssDNA, such as radioactive labeling or asymmetric PCR, but there are disadvantages in their use such as the purification time necessary for the strand's separation or the limitations due to radioactivity contamination, therefore, the development of new approaches for the ssDNA production is desirable to be able to make the methodology more efficient.

2.2 Hypothesis

Faced with this problem, we ask: how can we improve the detection of pathogens in the field of aquaculture (and food industry in general)?
To answer this question, we hypothesize:
Pathogenic bacteria present in food industry and aquaculture samples of interest could be quickly detected with the help of aptamers.

Although aptamers are not able to provide us with specific information about the target, such as its molecular mass or other particular characteristics as some other laboratory analysis methodologies do, aptamers can be developed with high specificity and sensitivity against the ligand, which allows to identify it in a precise and timely manner.

As for finding a solution for ssDNA production, we hypothesize that:
The use of nicking enzymes could improve the methodology by accelerating handling times.

2.3 Objectives

In order to verify the hypothesis, the following objectives have been set:

- General objective: Selection of aptamers to identify pathogenic bacteria of interest. To achieve this objective, different ligands of interest have been selected: two whole bacteria with an important presence in the food industry (*Salmonella* and *Campylobacter*), and a whole bacteria with an important presence in the aquaculture industry (*Vibrio parahaemolyticus*), which is the cause of important economic losses in shrimp farming. In addition, the PirAB complex has been selected: a toxin formed by two protein subunits that causes illness in shrimp. Using SELEX, some candidate aptamer sequences have been generated using various methods for generating single stranded DNA.

Specific objectives: to achieve the validation of the general objective, the following two specific objectives have been set:

- To improve the generation of single stranded DNA using various combinations of nicking enzyme digestion and purifications in order to accelerate the *in vitro* selection process, using different combinations of approaches to the use of enzymes un different SELEX
- To select specific aptamer sequences for the detection of the ligands of interest. To achieve this objective, the enzymatic methods of the previous objective will be used for the generation of ssDNA within SELEX for several targets.

3 MATERIALS AND METHODS

3.1 Improvement of the ssDNA production method for SELEX

3.1.1 Library design with restriction sites

The selection process for aptamers has started with a random library of 10^{16} DNA sequences synthesized by *Alpha DNA* and *Integrated DNA Technologies (IDT)*. For its design, the restriction sites of three nicking enzymes and one restriction enzyme have been selected, with the purpose of making strategic cuts that allow obtaining ssDNA. **Table 3-1** shows the design of the library used, consisting of a forward primer (FW) of 22 nucleotides, a reverse primer (RV) of 20 nucleotides and a section of 50 random nucleotides.

Table 3-1 Library design

5′ Cy5 TTGGTACCTCAGCTATGCAGTG RANDOM SEQUENCE (50nt) AGAGAGACGGTCCTGCAGAC 3′ 3′ AACCATGGAGTCGATACGTCAC RANDOM SEQUENCE (50nt) TCTCTCTGCCAGGACGTCTG FAM 5′					
1- KpnI	2 - Nb. BbvCI	3 - Nb. BtsI		4 - Nt. BsmAI	5 - PstI-HF

Table 3-1 shows the restriction sites for nicking and restriction enzymes.

Primers with different modifications were used during the amplification, depending of the digestion type; for lambda digestion a phosphorylation is necessary on the reverse strand to allow the enzyme to recognize the strand to degrade, for nicking digestion fluorescence has been added to the DNA to monitor the different enzyme's cuts. **Table 3-2** shows the different primers.

Table 3-2 Different primers used

Primer	Modification	Use
FW: TTGGTACCTCAGCTATGCAGTG	5' Cy5	Nicking digestion
RV: GTCTGCAGGACCGTCTCTCT	5' FAM	Nicking digestion
FW: TTGGTACCTCAGCTATGCAGTG	None	Lamba digestion
RV: GTCTGCAGGACCGTCTCTCT	5' Ph	Lamba digestion

3.1.2 Double stranded DNA production

For double stranded DNA production, a polymerase chain reaction (PCR) has been carried out with the following final concentrations: 0.5 µM of each primer, 200 µM of each dNTP, 1 X of PCR buffer, 1 U of Accustart II Taq DNA Polymerase and 0.005 µM of DNA template (SELEX library) in a final volume of 50 µl. The conditions used are shown in **Table 3-3**.

Table 3-3 Condition of PCR reaction

Phases	Conditions	Number of cycles
Initial denaturation	94°C-3 min	
Denaturation	94°C- 30 sec	25 cycles
Hybridation	58°C-30 sec	
Elongation	72°C-30 Sec	
Final elongation	72°C-3 min	

3.1.3 New generation of ssDNA using nicking and restriction enzymes

This method is part of our contribution to "Novel Strategies to Optimize the Amplification of Single-Stranded DNA" from Frontiers (Nehdi, et al., 2020).

For single enzyme digestion, the reaction was performed as suggested by the manufacturer (NEB). For multiple enzyme digestion (KpnI, Nb.BsmAI, Nb.BtsI, Nb.BbvCi, and PstI), 1 µg of DNA amplified fragments were digested by 10 U of each enzyme and 1x of CutSmart buffer in a final volume of 50 µl at 37 °C for 1 h with 1X NEB Buffer 1.1 that contains (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 100 mg/ml BSA at pH 7). Then the digested fragments were heated to 80 °C for 20 min for denaturation, and then chilled to 4 °C. Note that all the chosen enzymes recognize a single sequence, ranging from five bases (Nt. BsmAI: 50-GTCTC-30) to seven bases (Nb. BbvCI: 50-GCTGAGG-30), see Table 3-1.

The digestion of fluorescent labeled DNA was heated, or not, 10 min at 94 °C for denaturation and DNA complexes from all reactions were separated by native electrophoresis in a room at 37 °C on a 3% agarose gel. Fluorescence was imaged with a Typhoon FLA9500 (Nehdi, et al., 2020).

3.1.4 Nicking endonuclease digestion

For samples digested by nicking endonuclease, the following protocol has been performed:

1 µl of each enzyme (**Table 3-1**) (New England BioLabs) per 1 µg of dsDNA, 5 µl of cutSmart buffer (New England BioLabs), water to 50 µl. Incubation of 60 minutes at 37°C, inactivation of 5 minutes at 94°C.

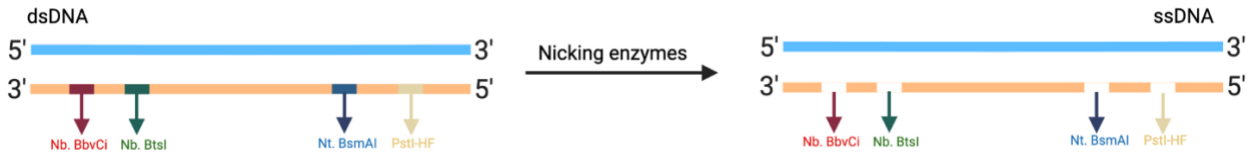


Figure 3-1 Nicking digestion

Figure 3-2 shows the recognition method used by nicking enzymes.

After digestion, heating to 94°C for 5 minutes is required in the case where gel purification is not carried out.

3.1.5 Lambda exonuclease digestion

After PCR, dsDNA was digested by lambda exonuclease (New England BioLabs); adding 3 µl of enzyme per 50 µl of PCR product, with an incubation of 30 minutes at 37 °C and an inactivation of 10 minutes at 80 °C. **Figure 3-1** shows the enzymatic action.

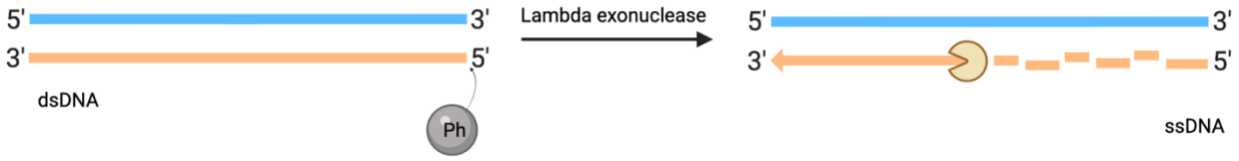


Figure 3-2 Lambda digestion

Figure 3-1 shows the recognition method used by lambda exonuclease.

3.1.6 Digestion product verification by polyacrylamide gel electrophoresis (PAGE)

A gel verification has been carried out with the nicking digestion products by acrylamide gel. A denaturing acrylamide gel 6% has been prepared with 12 ml of acrylamide 20% 8 M urea, 28 ml of 8 M urea TBE 1X (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8), 320 µl of APS 10% and

16 µl of TEMED. The samples were run with 2x denaturing dye (0.05% bromophenol blue, 0.05% xylenecyanol blue, 10 mM EDTA in formamide) 1 hour at 500 V and analyzed with a Typhoon FLA 9500 (GE healthcare Life Sciences) with two filters; due to the presence of two different fluorophores in the sample (**Table 3-2**) to verify the correct cutting of enzymes in function to the size.

3.1.7 Summary of enzymatic digestion combinations used in the *in vitro* selection process

Table 3-4 shows the different enzymatic digestion combinations used during the *in vitro* selection to test their efficiency and be able to select the best way to improve the methodology.

Table 3-4 Summary of enzymatic digestion combinations

Target	Total number of cycles	Cycles performed with PAGE purification	Cycles performed with nicking digestion	Cycles performed with lambda digestion
Vibrio parahaemolyticus (strain EB101)	12	All	All	0
Vibrio parahaemolyticus (strain EB101)	12	All	All	0
Salmonella enterica sero enteritidis	15	Cycle 6 only (before changing digestion method)	Cycle 1 to 6	Cycle 7 to 15
Salmonella enterica sero. enteritidis	15	0	0	All
Campylobacter jejuni sub. jejuni	15	Cycle 7 to 14	All	0
Campylobacter jejuni sub. jejuni	15	Cycle 7 to 14	Cycle 7 to 14	Cycle 1 to 6
Complex PirAB	14	0	0	All

3.2 SELEX against pathogenic bacteria

3.2.1 Library SELEX

The *in vitro* selection has been carried out with the library designed in section 3.1.1 against different targets; for food industry *Salmonella enterica* sero. enteritidis and *Campylobacter jejuni* sub. jejuni were selected for their incidence in food contamination, for aquaculture industry *Vibrio parahaemolyticus* and its toxin, the PirAB complex were selected due to the disease (AHPND) that they cause in shrimp cultures.

3.2.2 PirAB toxin

The complex PirAB toxin that we used was purified in UNAM (Universidad Nacional Autónoma de México), at Chemistry Institute in collaboration with CIAD (Centro de Investigación en Alimentación y Desarrollo) and stocked in phosphate buffer 50 mM, pH 7.5 with 150 mM NaCl.

PirA construction

PirA = rHTPirA (H = HisTag, T = cutting site TEV)

MGSSHHHHHHSSGENLYFQ**GHMSNNIKHETDYSHDWTVEPNGGVTEVDSKHTPIIPEVGRSV DIENTGRGELTIQYQWGAPFMAGGWKVAKSHVVQRDETYHLQRPDNAFYHQRIVVINNGAS RGFCTIYYH**

Number of amino acids: 132

Molecular weight: 15077.53 Da

PirB construction

PirB = rTrxPirB (Trx = thioredoxin, to which is merged PirB)

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNP GTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLVP RGSGMKETAAAKFERQHMDSPDLGTDDDDK**AMGTNEYVVTMSSLTEFNPNNARKSYLFDNY EVDPNYAFKAMVSFGLSNIPYAGGFLSTLWNIFWPNTPNEPDIENIWEQLRDRIQDLVDESIID AINGILDSKIKETRDKIQDINETIENFGYAAAKDDYIGLVTHYLIGLEENFKRELDGDEWLGYAIL PLLATTVSLQITYMACGLDYKDEFGFTDSDVHKLTRNIDKLYDDVSSYITELAAWADNDSYNN TNQDNVYDEVMGARSWCTVHGFEHMLIWQKIKELKKVDVFVHSNLISYSPAVGFPSGNFNYI ATGTEDEIPQPLKPNMFGERRNRIVKIESWNSIEIHYYNRVGRLKLTYENGEVVELGKAHKYD**

EHYQSIELNGAYIKYVDVIANGPEAIDRIVFHFSDDRTFVVGENSGKPSVRLQLEGHFICGMLA DQEGSDKVAAFSVAYELFHPDEFGTEK

Number of amino acids: 598

Molecular weight: 67353.40 Da

3.2.3 Optimization of target immobilization protocol

CNBr magnetic beads

We have decided to use magnetic beads as a means of ligand immobilization. Indeed, previous SELEX against bacteria performed in the laboratory faced several problems related to simple centrifugation, such as some candidate loss by using centrifugation as a separation method in positive selection.

CNBr activated SepFast MAG (BioToolomics) is a magnetic agarose between 50 – 150 µm that can bind small and large ligands in presence of a NH_2 group, **Figure 3-3** shows the ligation reaction used by CNBr beads.

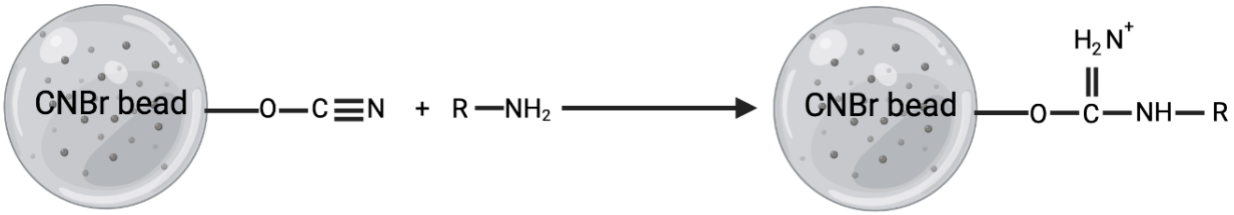


Figure 3-3 CNBr ligation

CNBr reaction was optimized for whole bacteria as a ligand SELEX, tested with different incubation times and different concentrations of target in order to cover the entire surface of magnetic beads, **Figure 3-4** shows the final result after the optimization protocol.

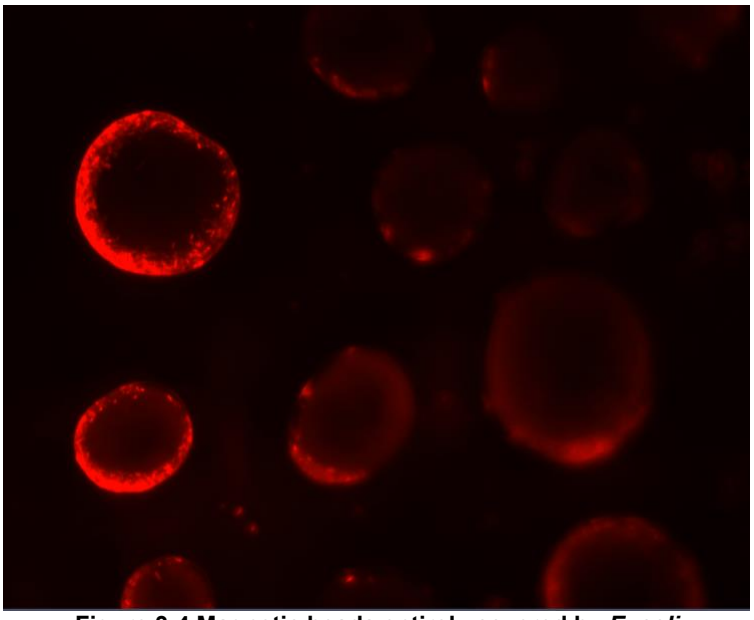


Figure 3-4 Magnetic beads entirely covered by *E. coli*

Magnetic beads (50-150 nm) conjugated with *E. coli* producing mCherry, used for optimization.

Optimized protocol

0.02 g of dry powder of CNBr has been activated with 200 µl (volume variable according to the SELEX cycle, **Table 8-1, Appendix I**) 1 mM HCl (previously refrigerated overnight) for 30 minutes at room temperature, after a HCl washing, the gel is mixed with the ligand (O.D. = 2.2, D. F. = 1000), diluted in 1 ml of coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl pH 8.3), incubated 2-3 hours in rotation at room temperature (vortexed each 30 minutes). After incubation three washes are necessary with 1 ml coupling buffer. To block the reaction, an incubation with 1 ml of blocking solution (0.1 M Tris/HCl pH 8.0) was done for 1-2 hours, in rotation at room temperature, the washed with 5 volumes of 0.1 Tris/HCl + 1 M NaCl pH 8.0, washed with 5 volumes of 0.1 M acetate buffer + 1 M NaCl pH 4.0, and a final wash with working buffer (PBS 1X, $MgCl_2$ 5 mM) was done.

Nanomagnetic particles

Nanoparticles (NHS-NH_2) of 200 nm were used to bind the PirAB complex with the following protocol:

Mix the following final concentrations: nanomagnetic particles 5%, 0.3 mg/ml ligand, coupling buffer to 1 ml. Incubation 1 hour, in rotation at room temperature, washing with 1 ml of coupling

buffer two times, add 1 ml of blocking solution and incubation 1 hour, in rotation at room temperature. Washing with 1 ml 0.1 M Tris/HCl (pH 8.0) + 1 M NaCl, washing with 1 ml of acetate buffer + 1 M NaCl pH 4.0 and a final wash with 1 ml of toxin working buffer (20 mM Tris/HCL, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4)

3.2.4 *In vitro* selection

- Negative selection

Some negative selections were done to improve the aptamers specificity, after the firsts three SELEX cycles when the amount of DNA has replicated enough to have a number of enriched copies. Each negative selection was performed before positive selection in order to separate the DNA binding to the negative target and continue with the enrichment (details in **Table 8-1, Appendix I**).

- Positive selection

During each SELEX cycle (**Figure 3-5**) ssDNA was incubated with 700 µl of whole bacteria linked to magnetic beads (**section 3.2.2**) in 700 µl of working buffer during an incubation time of 1 hour, in rotation at room temperature (variable time, **Table 8-1, Appendix I**).

The ligand was immobilized with the help of a magnetic support and the supernatant was separated. Two washes were done with 700 µl of working buffer and the positive selection product was resuspended in 100 µl of water.

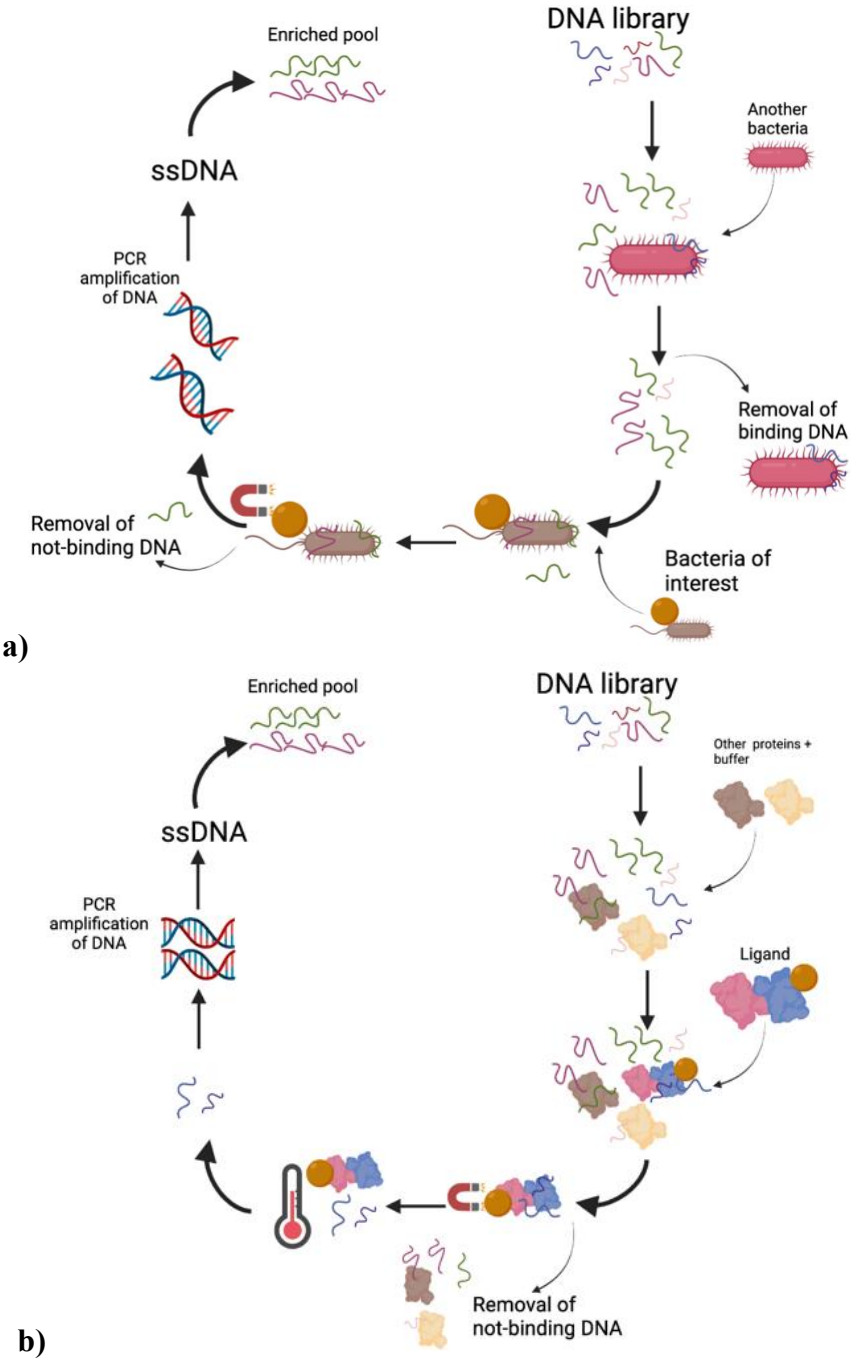


Figure 3-5 Full SELEX cycle

a) SELEX cycle with whole bacteria as a ligand. b) SELEX cycle with PirAB complex as a ligand.

3.2.5 PCR amplification

For the first cycle of SELEX all positive selection products were amplified with the PCR protocol of **section 3.1.2** (variable volume, **Table 8-1, Appendix I**).

3.2.6 Single stranded DNA production

- Lambda exonuclease digestion

ssDNA production with lambda exonuclease was performed with the **section 3.1.3** protocol.

- Nicking endonucleases digestion

ssDNA production with nicking endonucleases was performed with the **section 3.1.4** protocol.

3.2.7 Denaturing polyacrylamide gel purification

All the digestion products were separated by size in a 6% Polyacrylamide Gel Electrophoresis (PAGE) (**section 3.1.5**) based on the SELEX type (**Table 8-1, Appendix I**).

After migration, an elution of the samples of interest was done by putting the polyacrylamide band in 500 µl of elution buffer (0.3 M NaCl) overnight, and an ethanol precipitation was performed by adding 0.1 volume of 3 M NaCl (vortexed 10 sec), 2.2 volume of ethanol 95% (vortexed 10 sec) and left to precipitate 1 hour at -80°C. Then, tubes were centrifuged at 21 000 g / 20 minutes / 4°C, washed with 500 µl ethanol 70%, centrifuged at 21 000 g / 5 minutes / 4°C, dried at room temperature / 2 hours, and resuspended in Milli-Q water.

3.2.8 Sequencing and bioinformatic analysis

Following SELEX, some generations were sequenced (**Table 3-5**) by Illumina technology at Centre d'innovation Génome Québec and Université du Québec à Montréal.

Table 3-5 Generations sequenced

SELEX against:	Generations sequenced
Vibrio parahaemolyticus	6, 8, 12
Salmonella enterica sero enteritidis	5, 10, 15
Campylobacter jejuni sub. jejuni	5, 10, 15
Complex PirAB	6, 10, 14

The results were analyzed by the software Aptasuite (Hoinka, Backofen, & Przytycka, 2018) to align the different sequences and determine their abundances in each generation.

3.2.9 Affinity test

Vibrio parahaemolyticus

AFFINITY PRELIMINARY TEST

Different solutions in working buffer with different fluorescent candidate aptamer concentrations were done (0.01, 0.03 and 0.1 µM) and the ligand condition of the last cycle (**Table 8-1, Annex I**). Incubation of 15 minutes at room temperature was carried out followed by a wash and a final resuspension in 100 µl of working buffer. A sample measurement was done in Tecan INFINITE M1000 PRO with a wavelength of 646 nm for excitation and 662 nm for emission for Cy5 fluorescence.

SPECIFICITY PRELIMINARY TEST

The same preliminary test was performed but using ligands used in the negative selections during SELEX, in order to check the candidate's specificity.

AFFINITY PERCENTAGE TEST

Two candidates (**Table 3-6**) were selected from the preliminary test and synthesized by IDT. In order to verify the affinity percentage, the same test was performed but this time a measurement with a Typhoon FLA 9500 (GE healthcare Life Sciences) was done.

Table 3-6 Sequences selected for *Vibrio parahaemolyticus* candidat aptamers

SELEX type (Nicking digestion)	Candidat number	Candidat
PAGE	0	TTGGTACCTCAGCTATGCAGTGGGGTGTGTTTAGCCGAAGCCGTCAGTGCAATGCCACCGTCTTGGTGTTGTAGAGAGACGGTCCTGCAGAC
Heating	4	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACAGAGAGACGGTCCTGCAGAC

PirAB complex

A sandwich test was performed (**Figure 3-6**); one version of each aptamer was modified with a NH_2 molecule to bind it to magnetic nanoparticles and then with the PirAB complex, after a second version of each aptamer modified with Cy5 fluorescence was put in contact with the magnetic bead–NH_2 aptamer-PirAB complex construction and a Typhoon measurement was done in order to identify the Cy5 fluorescence and quantify the affinity percentage.

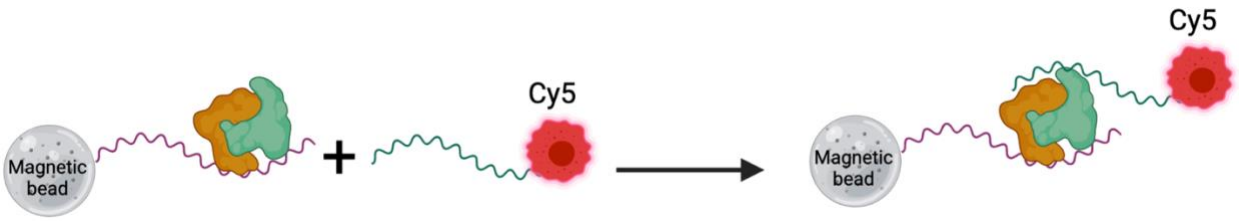


Figure 3-6 Sandwich test principle

For NH_2-modified aptamers, ligation to nanoparticle beads was performed with the **section 3.2.2** protocol with an aptamer concentration of 1 mg/ml. To finish the magnetic bead–NH_2 aptamer-PirAB complex preparation, a toxin concentration of 0.3 mg/ml was used (randomly chosen due to the limited amount of purified complex available) in the mix and incubated for 15 minutes, in rotation at room temperature. Once the construction was done, 5 pmoles of the fluorescent version of aptamers were added according to combinations presented in **Table 3-6**.

Table 3-7 Sandwich test relation

Aptamer version	Aptamer candidate				
NH_2	1	2	3	4	5
Cy5	2, 3, 4, 5	1, 3, 4, 5	1, 2, 4, 5	1, 2, 3, 5	1, 2, 3, 4

A total of 20 samples was tested to verify the relation between each aptamer version.

3.2.10 Microscale thermophoresis

The sequences selected by the sandwich test were synthesized *by Alpha DNA*, candidate 4 was modified with Cy5 fluorophore labeling for analysis by microscale thermophoresis. A concentration of 5 nM of candidate 4 was incubated with different concentrations of the PirAB complex (7.3 µM to 0.2 nM with a serial dilution by a factor of two) in toxin working buffer for 20 minutes and a subsequent incubation of 24 hours at 4° C for a correct reading by the Monolith NT.115 Pico device (NanoTemper technologies).

Samples of the serial dilutions were transferred to fine capillaries (NT.115 Standard Treated Capillaries) and a Cy5 fluorescence measurement was performed. Finally, the device generated a report with saturation curves and a K_D value for the analyzed candidate.

4 RESULTS

4.1 Improvement of the selection method of SELEX

There exist numerous versions of SELEX, most alternative methodologies are modifications of the selection step. However, there are also several methods to produce ssDNA, each with advantages and disadvantages. One of them is with the lambda nuclease, which can degrade the reverse strand if it is 5´ phosphorylated. This method is however not suitable in some cases, for instance if we require the forward strand to have a 5´ phosphate for a ligation step. For that reason, we explored another way to produce ssDNA with nicking enzymes.

4.1.1 New generation of ssDNA using nicking and restriction enzymes

The enzymatic action was tested individually in order to evaluate the action of each nicking enzyme, as well as with a combination of all the enzymes (**Figure 4-1**). This allowed gel-based separation of forward (uncleaved) vs reverse (cleaved) for both the PCR product where a template with a random region was used, as well as with a template made up of a constant sequence.

These results are part of our contribution to:
Nehdi N, Samman N, Aguilar- Sánchez V, Farah A, Yurdusev E, Boudjelal M and Perreault J. (2020) Novel Strategies to Optimize the Amplification of Single-Stranded DNA" from Front. Bioeng. Biotechnol. (Nehdi, et al., 2020).

Most restriction enzymes are very specific for their cognate site, but because they cleave both strands, they would provide only a small size change as compared to the complementary strand. Conversely, nicking enzymes cleave only one strand, while still being very specific for their cognate site. We thus included nicking restriction sites in the constant sequences of the primer binding sites of our library; for our templates we used two long oligonucleotides of 92 nt, both a constant sequence (**Figure 4-1** A, top) and an oligonucleotide with a randomized middle region (**Figure 4-1** A, bottom). All three nicking enzymes cleaved the reverse strand (**Figure 4-1** B, RP label, lines 2-4), while leaving the forward strand intact (**Figure 4-1** B, FP label, lines 2-4). Even if the gel pictured in **Figure 4-1** B is a 20% denaturing PAGE, separation of forward strand and reverse strand is relatively easy. As noted by the smaller digestion products when enzymes are

combined (**Figure 4-1** B, RP label, lane “all”), the size difference is even larger in this case. Additional “standard” restriction enzymes (PstI-HF and KpnI) were included as well to provide further fragmentation potential of the constant DNA sequence, as well as allow ligation and cloning if necessary. Note that even if in principle using a single nicking enzyme suffice to distinguish between the forward and reverse strands, as shown by differences in migration for bands with RP label compared to FP label (**Figure 4-1** B), using two enzymes, such as 2 and 3 (**Figure 4-1** B) will provide a larger size difference (92 vs. 50 C 22 C 20 bases) which will make it even easier to distinguish on gel. Furthermore, the native gel in **Figure 4-1** C, also corroborates the differences between fixed sequences and random libraries, even suggesting that denaturing gel purification might not be absolutely required for strand separation, as indicated by the unique band corresponding to FS ssDNA when the digested sample is heated (**Figure 4-1** C, random, dig, “H”) (Nehdi, et al., 2020).

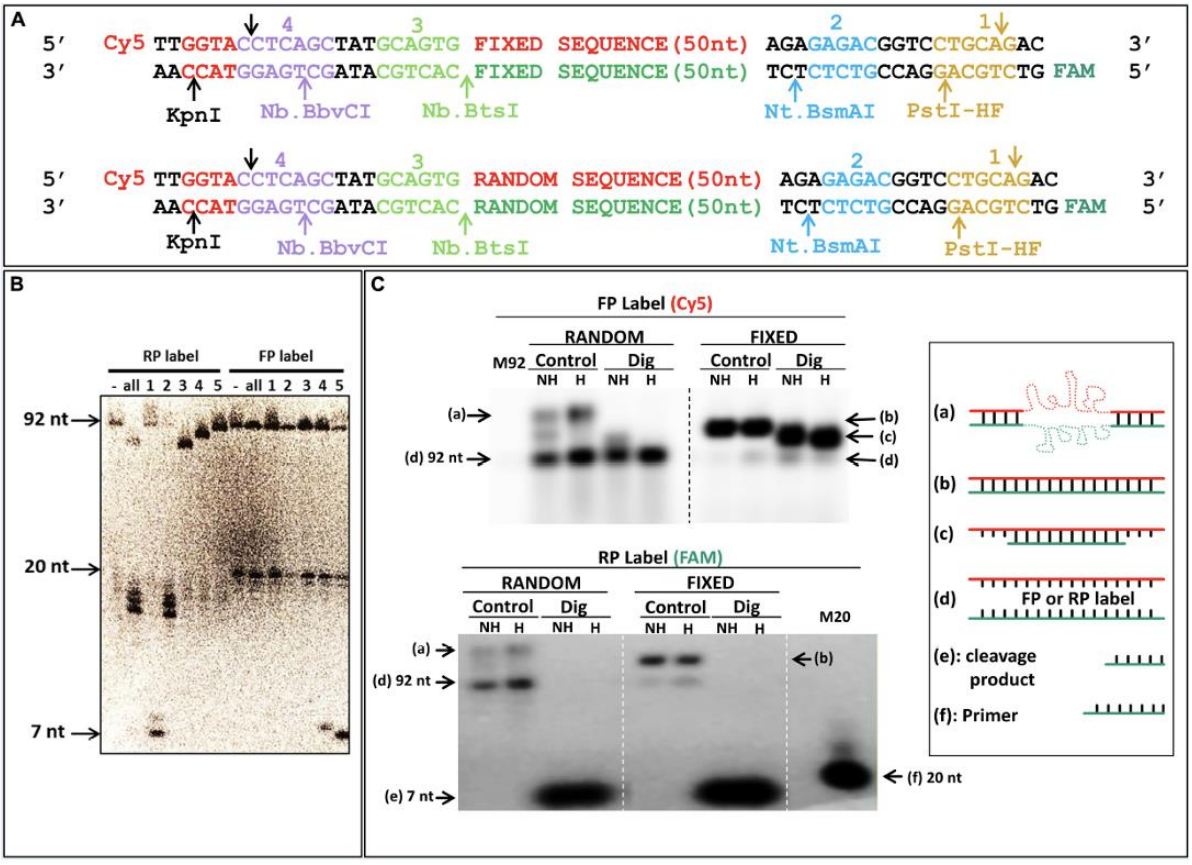


Figure 4-1 Specific digestion of reverse strand DNA

(A) Scheme of the designed library, and control “fixed” sequence, with cleavage sites of each enzyme pictured in a different color in the constant or random sequence regions with fluorescent labels; “Cy5”: cyanine (Forward); “FAM”: fluorescein (Reverse). (B) Denaturing urea PAGE of digested DNA. Numbered sites in (A) correspond to the enzymes

used in the lanes of the gel; RP label and FP label: radioactive labeling of reverse strand via the labeled reverse and forward primers, respectively; "-": uncleaved PCR; "all": all three nicking enzymes (2, 3, and 4) together with KpnI (5) or PstI-HF (1), in RP or FP label, respectively. Full length DNA is 92 bases; the 20 bases band correspond to labeled primers; and 7 bases band to either KpnI (FP) or PstI-HF (RP) cleavage products. (C) Native agarose gel of DNA with fixed and random sequences. Each PCR amplicon was labeled with fluorescence as indicated in (A) and imaged with a Typhoon FLA9500 for Cy5 (FP label) and fluorescein (RP label). M92: marker ssDNA, 92 bases; "M20" Marker ssDNA, 20 bases correspond to labeled primer; "Control": uncleaved PCR; "Dig": digestion with all three nicking enzymes (2, 3, and 4) together with PstI-HF, in reverse primer label; full length DNA is 92 bases; and 7 bases band corresponds to PstI-HF (RP) cleavage products; NH: samples were not heated before loading; H: samples were heated to 94 C before loading; "a–f": different structures of DNA and markers used are schematically represented (on the right side). Electrophoresis was done in a room at 37 C in a 3% NAGE (native agarose gel) Source: (Nehdi, et al., 2020)

In **Figure 4-1** C (top) we can see that the ssDNA produced by enzymatic digestion is much clearer (higher concentration) for the sequence samples with a randomized middle region than for the fixed sequence. This shows the fact that enzymatic digestion with nicking enzymes is much more efficient in generating ssDNA when we are in the presence of a sequence where the abundance of complementary fragments is lower than when the fragments can quickly locate their complementary section as it is for a sample with a high concentration of identical copies (i.e., with a fixed sequence).

4.1.2 Nicking endonuclease-based single stranded DNA production for SELEX

The use of endonucleases for the ssDNA production facilitates the digested strands separation in a random library where the diversity of the sequences is very large. However, as the enrichment progresses, this library diversity decreases (which also means that the number of copies of each sequence increases) and the probability that the digested fragments find their complementary DNA strand is greater.

During the SELEX methodology, two main strategies have been used; the enzymatic digestion with and without the gel purification step. Skipping the purification step or performing it in strategic generations has been a selection made in order to compare results due to the limitations observed, as mentioned above.

Some problems have arisen during the development of SELEX with ssDNA production by nicking enzyme digestion. After cycle 7, the product concentration of the expected size begins to decrease and the artefacts begin to appear in greater concentration (**Figure 4-2**), this tells us of a possible interference of the small DNA fragments digested. Indeed, we can easily imagine that

after a few cycles of selection, the reduced library diversity increased the probability of the complementary strand to interact with the forward strand, thus creating a selective pressure for shorter strands that would less readily anneal together.

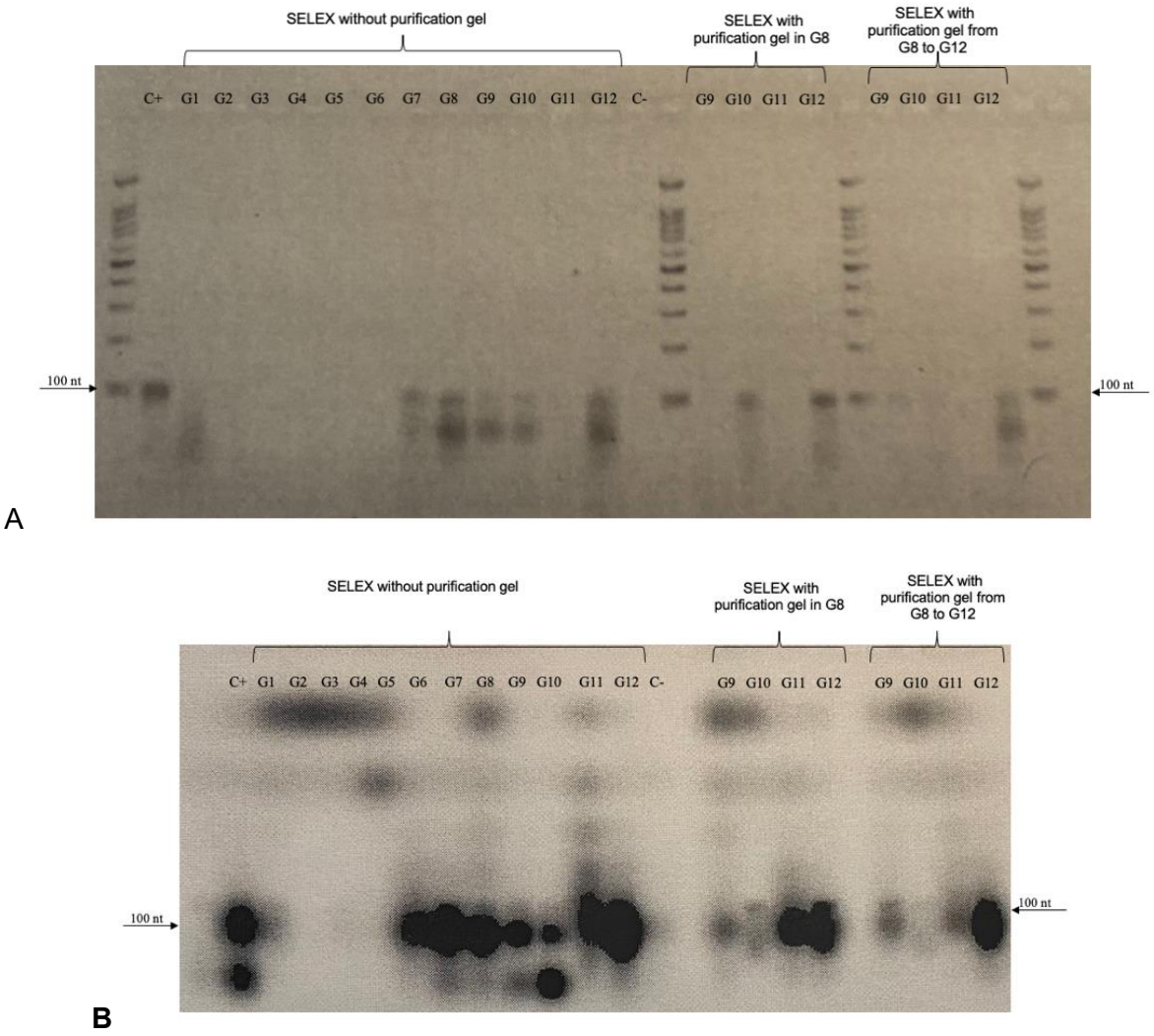


Figure 4-2 dsDNA product (amplified enriched libraries) Vibrio parahaemolyticus SELEX without purification gel

Agarose gel 2%, G1-G12= generation number, C+: amplified library, C-: PCR mix without Taq DNA Polymerase. A: scanned by GelDoc, B: scanned by Typhoon FLA9500

Once the problem of the appearance of artefacts was observed, a division of the methodology was carried out to analyze if the gel purification step is required throughout the last generations or only in some of them to filter-out shorter sequences, or other artefacts. As can be seen in **Figure 4-2**, the use of the gel purification step in the most advanced SELEX generations allows the reduction of the presence of artefacts, whether used only as a filter or for each SELEX cycle

in the latest generations. However, surprisingly, it appears that purifying sequences at every SELEX cycle had no beneficial impact compared to a single PAGE purification for cycle 8; and might even be deleterious as can be seen for the short amplicons from G12 when PAGE was used at each cycle.

Note that in some generations the band of the expected size is faint when imaged with the Geldoc, so a reading has been made with a Typhoon FLA9500 in which the appearance of the bands can be seen as the contrast is increased (Figure 4-2 B). This tells us of a low concentration of products in the samples, which could be due to several reasons: poor yield during PAGE purification or conditions of the different SELEX cycles, like: interference of the nicking enzymes fragments in the cycles with higher enrichment, where complementary sequences can potentially hybridize back on the forward strand.

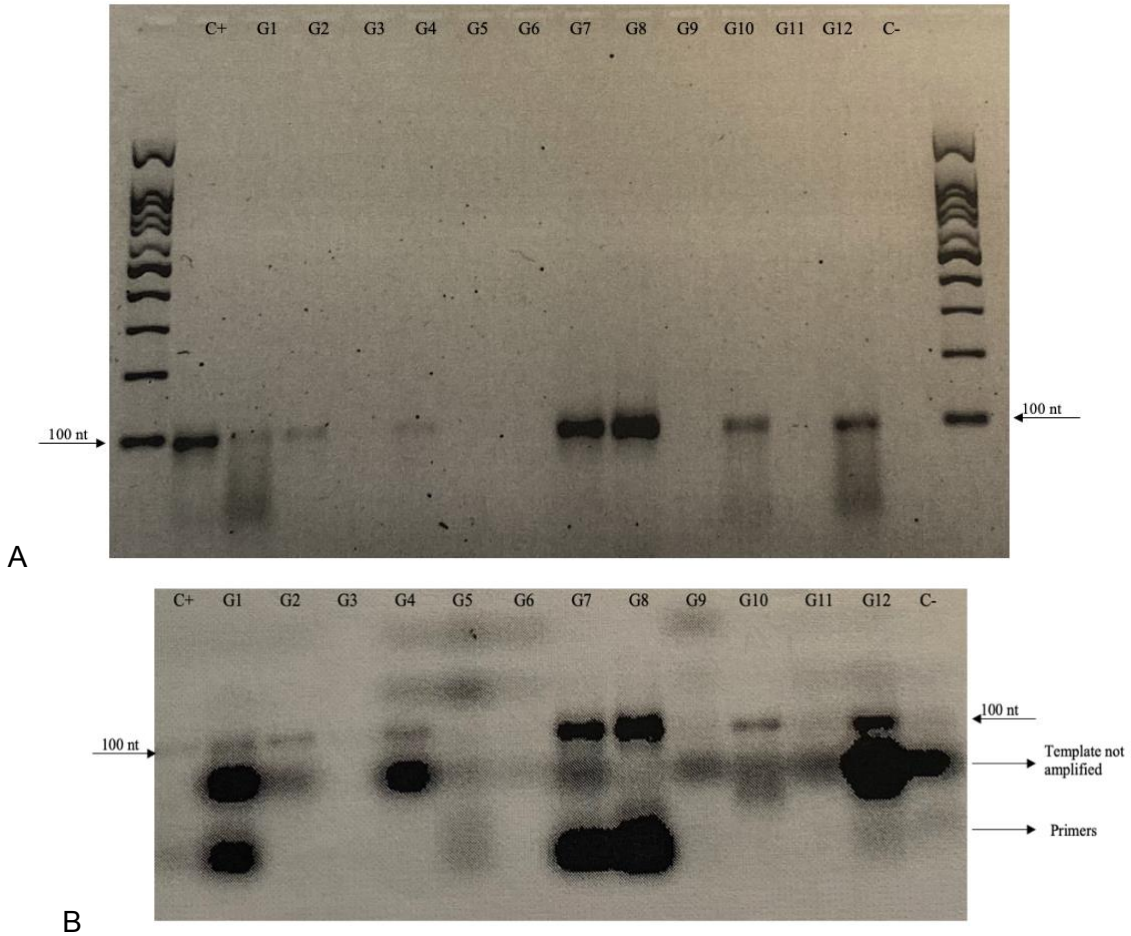


Figure 4-3 dsDNA product (amplified enriched libraries) *Vibrio parahaemolyticus* SELEX with purification gel,

Agarose gel 2%, G1-G12= generation number C+: amplified library, C-: PCR mix without Taq DNA Polymerase. A: scanned by GelDoc, B: scanned by Typhoon FLA9500.

As seen in **Figure 4-3**, and for the SELEX cycles shown in on the right part of the gel if **4-2**, short-PCR product artifacts are much less present with the use of the gel purification step.

Note that in **Figure 4-3** we can observe the presence of samples G3, G5, G6, G9 and G11, which are not noticeable in the GelDoc reading. This shows us a presence of the samples but in lower concentration.

4.1.3 Single stranded DNA production by lambda exonuclease digestion

The SELEX done with only lambda-based ssDNA production has shown amplified enriched libraries (PCR product) throughout the entire SELEX (**Figure 4-4**), albeit with relatively low intensity. This may be an indication that digestion has been carried out correctly and that the enzymatic action allows the generation of the ssDNA without interference of its complementary strand. In addition, all the methodology has been completed in 6 days; using this enzyme allows cycle acceleration to identify potential candidates faster.

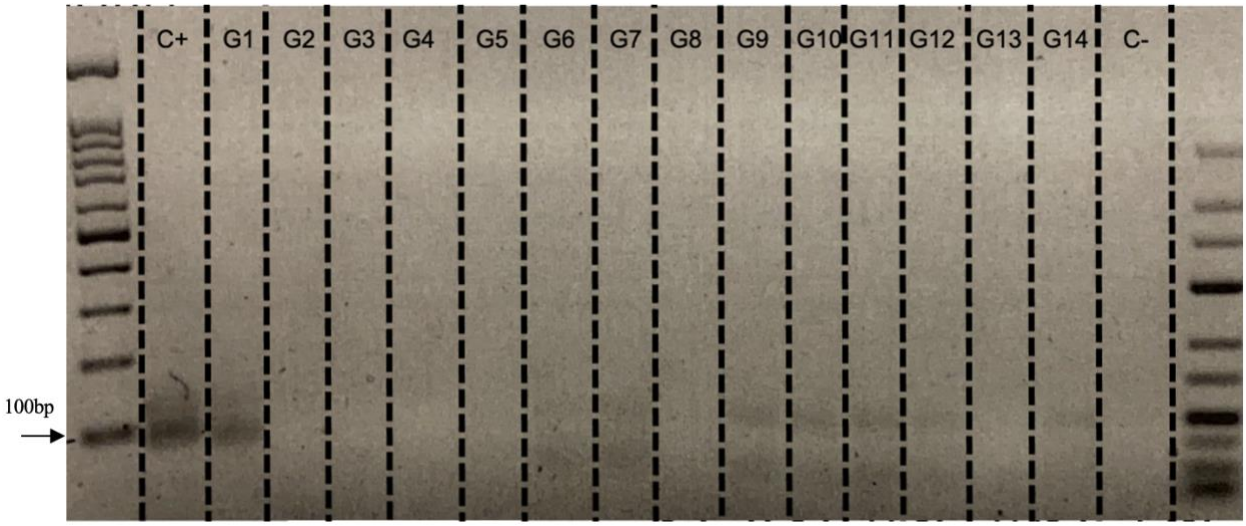


Figure 4-4 PCR product (amplified enriched libraries vs *PirAB*)

Agarose gel 2%. C+: positive control (amplified library), G= generation, 1-14= generation number, C-: negative control (PCR mix without template).

Note that in G2 and G3 the amount of ligand decreased considerably as a selection condition, G4 and G5 were exposed to the first negative selection with different ligands (magnetic beads and Lysozyme/BSA respectively); it is thus normal to see only faint bands on these samples.
In addition, an elution of the candidates has been used by heating the samples, from this elution the PCRs have been carried out, for which it is normal that in G8 and G13 we see that the

candidate's concentration has decreased considerably since the ambient temperature was not controlled after elution, it means that as there is a decreased diversity of the sequences due to enrichment, it is very likely that the eluted DNA has rapidly re-paired with the ligand and this did not allow adequate use of the template.

A PAGE verification was done in G6 in order to check the presence of ssDNA product at the expected size (**Figure 4-5**).

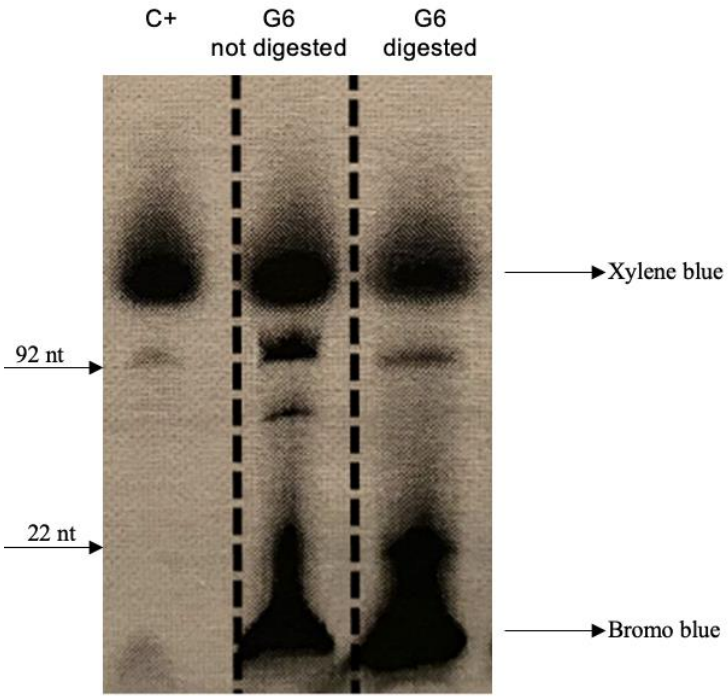


Figure 4-5 G6 verification

Denaturing acrylamide gel 6%, C+: single strand oligonucleotide of 92 nt with Cy5

The presence of the product was verified and the cycles have continued under normal conditions, without gel purification.

4.2 SELEX against pathogenic bacteria

It should be noted that the objective of carrying out the SELEX methodology on various ligands, in addition to comparing the different strategies of ssDNA generation, has been to obtain aptamers for ligands of interest in aquaculture and food safety.

4.2.1 Candidates for aptamers obtained against whole bacteria

SELEX have been performed for three different ligands where the target is the whole bacterium: *Vibrio parahaemolyticus, Salmonella enteric* and *Campylobacter jejuni.*

Regarding *Vibrio parahaemolyticus*, the methodology has been divided into four strategies; with and without gel purification step, with a filter-like purification in G8 and with purification from G8 to G12.

For *Salmonella enterica*, the SELEX methodology was used with two enzymatic digestion strategies; one in which lambda exonuclease has been used throughout the complete methodology and another in which nicking endonucleases have been used in the first six SELEX cycles and concluded with the use of lambda exonuclease.

For *Campylobacter jejuni*, SELEX was performed with the following ssDNA production strategies; using nicking endonucleases in all the SELEX cycles and with a gel purification step starting from G7 and a last strategy where lambda exonuclease-based ssDNA production started up to G6 and the SELEX cycles were finished with nicking endonuclease-based ssDNA production with a gel purification step.

The candidates have been selected based on the appearance of two or more copies during the bioinformatics analysis of the sequencing results and the size of interest to be characterized individually.

Vibrio parahaemolyticus

13 candidates were selected after bio informatic analysis from different SELEX types (**Table 4-1**) according to their abundance (i.e. the corresponding number of reads obtained by deep sequencing). For the preliminary affinity and specificity tests of these aptamers, the percentage of fluorescently tagged aptamers was obtained after binding assays with bacteria; and quantified with the ImageJ software (**Figure 4-6**). For these, PCR was performed using a reverse primer with fluorescein (FAM) label in 5′ and a forward primer with a Cy5 in 5′, thus allowing the production of fluorescently labeled aptamers from template sequences ordered from AlphaDNA. Two candidates with the best affinity percentage were selected after the preliminary tests (candidate 0 and 4) for complete chemical synthesis and Cy5 labeling (IDT) and assays to estimate their affinity were performed (**section 3.2.9**, affinity percentage test).

Table 4-1 *Vibrio parahaemolyticus* aptamer candidates selected

SELEX type (Nicking digestion)	Candidate number	Candidate
PAGE	0	TTGGTACCTCAGCTATGCAGTGGGGTGTGTTTAGCCGAAGCCGTCAGTGCAATGCCACCGTCTTGGTGTTGTAGAGAGACGGTCCTGCAGAC
	1	TTGGTACCTCAGCTATGCAGTGCCGACCCGACTGACAATCCTTTGCCCATTTCCATACACACCCCCGCTTCCAGAGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGCACACACCTGTCCACCCGCTACACTTCCCACTCCTCATTACCCACCTTCCCAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGCCAACAATACGTTCCCTTCCCACTCCATTCACCTTCACCAACCCCTTGCCCAGAGAGACGGTCCTGCAGAC
Heating	4	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACAGAGAGACGGTCCTGCAGAC
	5	TTGGTACCTCAGCTATGCAGTGGGGTGTGTTTAGCCGAAGCCGTCAGTGCAATGCCACCGTCTTGGTGTTGTAGAGAGACGGTCCTGCAGAC
	6	TTGGTACCTCAGCTATGCAGTGTCAACCATCATATACCCAAAGTCCTGCTCCCATACTTCTACTCCTTAACCAGAGAGACGGTCCTGCAGAC

Heating/ 1 filter PAGE	7	TTGGTACCTCAGCTATGCAGTGCCATCAACACCCTACTCCCCTC TACATTCAACATCCCCACACTCCCGTTCCAGAGAGACGGTCCTG CAGAC
	8	TTGGTACCTCAGCTATGCAGTGCCACAGTCTACAAGCCTTTCCC ATCTGATTTACCTTTCTATCAACTCCTCAGAGAGACGGTCCTGCA GAC
	9	TTGGTACCTCAGCTATGCAGTGTCGGCATCACCCATACCCCTGT ACTATCACCTATATTCCGTTCATCATCTAGAGAGACGGTCCTGCA GAC
Heating/ last 4 cycles with PAGE	10	TTGGTACCTCAGCTATGCAGTGGGGTGTGTTTAGCCGAAGCCGT CAGTGCAATGCCACCGTCTTGGTGTTGTAGAGAGACGGTCCTGC AGAC
	11	TTGGTACCTCAGCTATGCAGTGCCGGCTCCCCGCTATGGAAGGT CATTAGCTCCGGCAAGCAATTAAGAACAAGAGAGACGGTCCTGC AGAC
	12	TTGGTACCTCAGCTATGCAGTGTCAGCCCCACTCTCAGTCCCTT ACCACACCAAGCCCTCCTCCCCCATTCCAGAGAGACGGTCCTG CAGAC

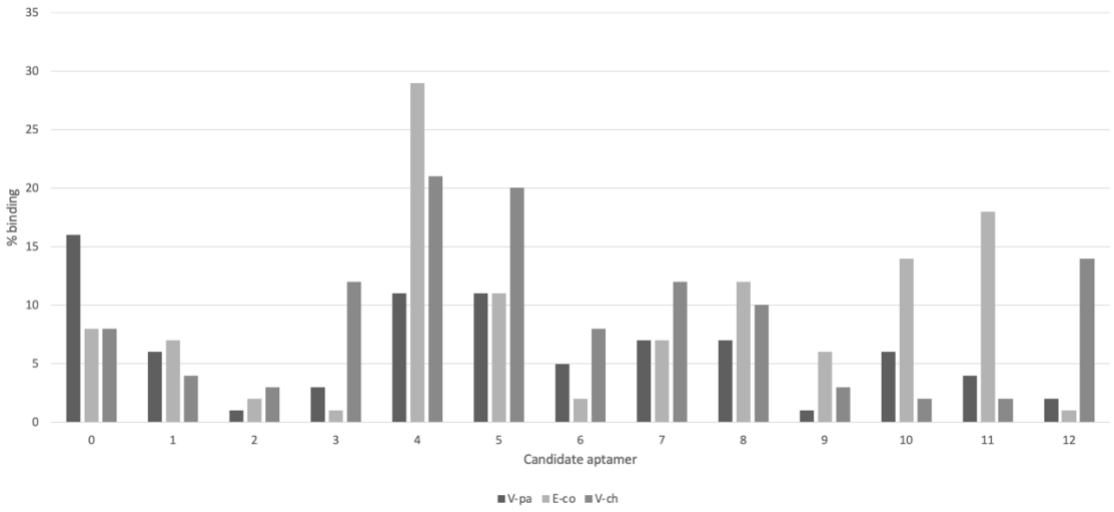


Figure 4-6 Affinity and specificity preliminary test

V-pa: *Vibrio parahaemolyticus*, E-co: *Escherichia coli*, V-ch: *Vibrio cholerae.*

Escherichia coli has been used as a specificity test and *Vibrio cholerae* for genus specificity. Note that the bacteria used as negative selection were: *Vibrio alginolyticus, Vibrio cholerae, Vibrio vulnificus* and *Salmonella enterica*, it is thus somewhat surprising to find *V. cholera* binding better to several aptamers. **Figure 4-7** shows the binding percentage obtained with the affinity test and a negative control against magnetic beads.

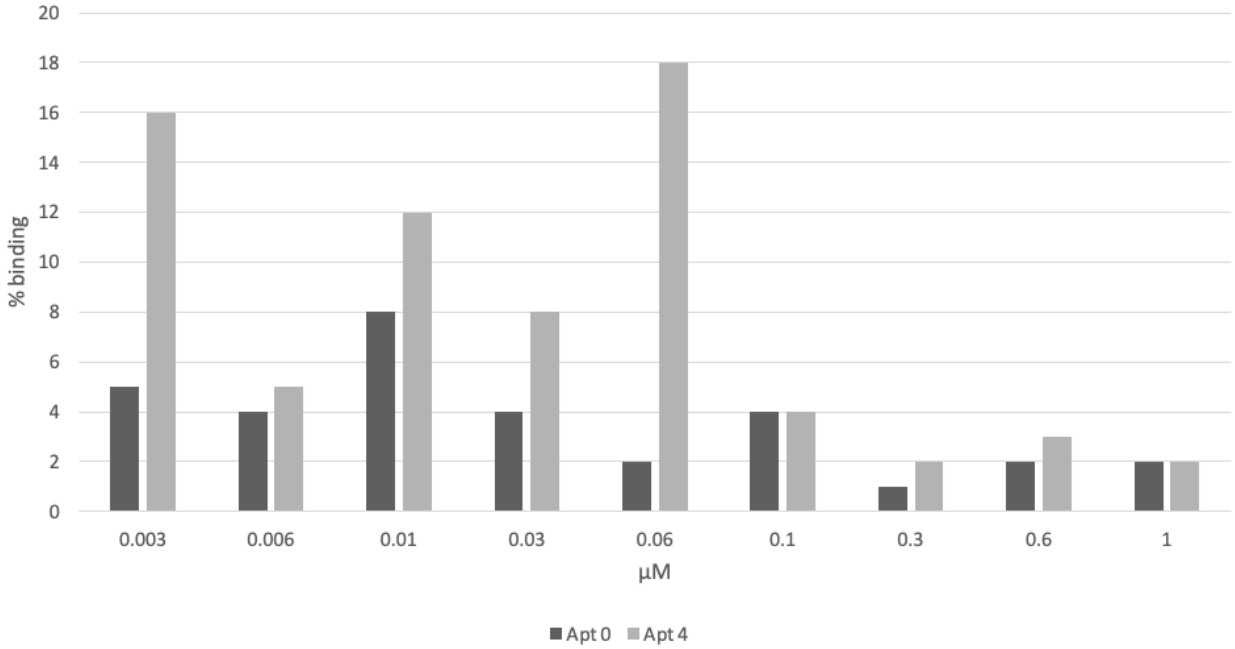


Figure 4-7 Binding percentage *Vibrio parahaemolyticus* candidate aptamers

	% ligation			
µM	Apt 0 to V-pa	Apt 0 to mb	Apt 4 to V-pa	Apt 4 to mb
0.003	5	4	16	8
0.006	4		5	
0.01	8	10	12	11
0.03	4	0	8	7
0.06	2		18	
0.1	4	2	4	3
0.3	1	0	2	1
0.6	2		3	
1	2	9	2	2

Figure 4-8 Candidate aptamers vs V-pa and mb

V-pa: *Vibrio parahaemolyticus*, mb: magnetic beads.

In **Figure 4-8** we can see that when performing the same test vs magnetic beads, the aptamer candidates yield binding signal results, which indicates a significant background noise in the binding results vs *Vibrio parahaemolyticus*.

Salmonella enterica sero. Enteritidis

As for *V. parahaemolyticus* , aptamer candidates were selected based on the deep sequencing results and Aptasuite analysis (based on their copies number and the sequences length) of the libraries derived from the *Salmonella enterica* sero. enteritidis selection (**Table 4-2**).

***Table 4-2 Salmonella enterica* sero. Enteritidis candidates**

SELEX type	Count	Candidate
Lambda digestion / not PAGE	23	TTGGTACCTCAGCTATGCAGTGGGCGGGGGGGGATGGGAAAGGTTT TCCTTTATGCATAGTGAGTTGTTTCTAGAGAGACGGTCCTGCAGAC
	19	TTGGTACCTCAGCTATGCAGTGCACACGAGTTGTGGGTGGGAGGGT GGCCGAGTGTGATATGACACGATGCTAGAGAGACGGTCCTGCAGAC
	9	TTGGTACCTCAGCTATGCAGTGCACATTACGCGCTTCTGGGAGGGT GGGTTGGGTTTCGAAGTGCGTCAGAGAGACGGTCCTGCAGAC
	8	TTGGTACCTCAGCTATGCAGTGTCAGCCCCACTCTCAGTCCCTTACC ACACCAAGCCCTCCTCCCCCATTCCAGAGAGACGGTCCTGCAGAC
	5	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACAGA GACGGTGTTGTAGAGAGACGGTCCTGCAGAC
	5	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACAGT GTCCCCCGTTCCAGAGAGACGGTCCTGCAGAC
	4	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACACA CCCGTTCCAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGCACACCCTACACATCGTACTCACCT CCTTATCCTCCCCCACCCCCATTCCAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACAGA GAGACGGTGTTGTAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGGGTGGGTGGGCGGCGTCATAGCAG TTAGTGGATTATCCCTTCGGTCGCTGTAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGCACACCTACCTACCCTCCACTTCTC TCACACTTCTCACACTTCCCATTCCAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGGGCGGGGGGGATGGGAAAGGTTTT CCTTTATGCATAGTGAGTTGTTTCTAGAGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACATCA GCATTCCCACCACCCGTTCCAGAGAGACGGTCCTGCAGAC
Nicking digestion + Lambda digestion / not PAGE	2	TTGGTACCTCAGCTATGCAGTGCCCACGATCCACACCACACAGTAAC TATTCTACTTCATTACTTCCCATTCCAGAGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGCCCCCCCATTCCCGACCATTTACCA ATTACCTACTCCTTTGGTCCCATTTCAGAGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGACAGA GACGGTGTTGTAGAGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGTCACACCCCTACCATCAGTCATCTC TAGCCCAACCCATCTCCCCATTCCAGAGAGACGGTCCTGCAGAC

A bioinformatic analysis of the sequencing results has been carried out with the Aptasuite software, through said analysis the sequences shown in **Table 4-2** have been selected based on the number of copies present in the results and their size. These sequences can be selected for further analysis of candidate aptamers and their characterization.

Campylobacter jejuni sub. jejuni

Table 4-3 shows aptamer candidates selected after bioinformatics analysis in Aptasuite against *Campylobacter jejuni sub. Jejuni* based on their copies number and the sequences length.

***Table 4-3 Campylobacter jejuni sub. jejuni* candidates**

SELEX type	Count	Candidate
Nicking digestion / PAGE (cycle 7-15)	6	TTGGTACCTCAGCTATGCAGTGTAGGTGCTGGGGATCCACTGAGAG AGACGGTCCTGCAGAC
	6	TTGGTACCTCAGCTATGCAGTGCAATGTGGGAATGTATTCATTAGTG AATTCCTTTCTTCACCTGTACTCAGAGAGAGACGGTCCTGCAGAC
	4	TTGGTACCTCAGCTATGCAGTGCCATGTGGGATTTGGTGTATTTAGT AATAAATTCCACCTTATGACAACACAGAGAGACGGTCCTGCAGAC
Lambda digestion + Nicking digestion / PAGE (cycle 7-15)	4	TTGGTACCTCAGCTATGCAGTGCCCCCGTTCCAGAGAGACGGTCCT GCAGACAGAGAGACGGTCCTGCAGAC
	3	TTGGTACCTCAGCTATGCAGTGCGAGAGAGACGGTCCTGCAGACAG AGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGTCACTCATCCTCCTCCATTGCTATAT CCTTCATTGTCCCACCCCCATTCCAGAGAGACGGTCCTGCAGAC
	2	TTGGTACCTCAGCTATGCAGTGCCACTCACTTTCACTCCTCCCCACA TCATACTATACCATCCTGCACCCTTAGAGAGACGGTCCTGCAGAC

In the same way as the *Salmonella* results, the *Campylobacter* SELEX sequencing results have been analyzed with the use of Aptasuite and the selected sequences are shown according to the copies present and their size (**Table 4-3**).

4.2.2 Candidates for aptamers obtained against toxin complex

Another SELEX has been carried out with the PirAB toxin as a ligand with the same objective of identifying *Vibrio parahaemolitycus*, a pathogen that produces this toxin causing cell detachment in the shrimp hepatopancreas, causing AHPND disease.

Five candidates for PirAB complex were selected from bio informatic analysis, based on their copies number and the sequences length. **Table 4-4** shows aptamer candidates selected after Aptasuite analysis

Table 4-4 PirAB complex candidates

SELEX type	Candidate number	Count	Candidate
Lambda digestion / not PAGE	1	30	TTGGTACCTCAGCTATGCAGTGTAGAGAGACGGTCCTGCAGA CAGAGAGACGGTCCTGCAGAC
	2	6	TTGGTACCTCAGCTATGCAGTGCCCCCCATCCTCCTTACAGCG CCCCCCACATCCTTACCACCCCATTCCAGAGAGACGGTCCTG CAGAC
	3	6	TTGGTACCTCAGCTATGCAGTGGGGGGAAGTACTTAGTGTGTT GTGGTAGAGAGACGGTCCTGCAGAC
	4	6	TTGGTACCTCAGCTATGCAGTGTCCGTACTTGGCATACCCCCC CACCTCCCTCCGCACTACCTACCCATTCCAGAGAGACGGTCC TGCAGAC
	5	6	TTGGTACCTCAGCTATGCAGTGTGGGACAAGGAAGTAATGTGT GTTGTGGTAAGAGAGACGGTCCTGCAGAC

4.2.3 Aptamer characterization

A sandwich test (**section 3.2.9**) was done for a preliminary characterization of PirAB complex candidate aptamers; based on the sandwich test results, there are two candidate aptamers (3N + 4C) that could work together to identify the toxin complex based on its reproducibility (**Figure 4-9**).

a)

	% linked MB+APT1
MixAPT	%
1N+2C	6%
1N+3C	13%
1N+4C	6%
1N+5C	0%

b)

	% linked MB+APT2
MixAPT	%
2N+1C	7%
2N+3C	3%
2N+4C	6%
2N+5C	2%

c)

	% linked MB+APT3
MixAPT	%
3N+1C	5%
3N+2C	3%
3N+4C	6%
3N+5C	2%

d)

	% linked MB+APT4
MixAPT	%
4N+1C	5%
4N+2C	6%
4N+3C	3%
4N+5C	5%

e)

	% linked MB+APT5
MixAPT	%
5N+1C	5%
5N+2C	2%
5N+3C	3%
5N+4C	4%

f)

% linked to MB	
APT	%
1	1%
2	6%
3	2%
4	1%
5	2%

Figure 4-9 Sandwich test results

xN/xC: x= candidate number, N=NH_2 aptamer version; C= Cy5 aptamer version; MB: magnetic beads.
a), b), c), d) and e) Candidate aptamer 1, 2, 3, 4 and 5, respectively, NH_2 version conjugated to magnetic beads and PirAB complex tested with all other Cy5 version candidate aptamers;
f) Negative control; all Cy5 version candidate aptamers tested vs magnetic beads.
*Note that all tests were done in duplicate, so no error bars are shown.

According to the results in binding percentage in the sandwich tests, the aptamer candidates 3N and 4C present a better response in the joint work of toxin identification, the results of the tests with both candidates together were similar and within the highest percentages of affinity, given

these results we consider both candidates as the most appropriate to continue the characterization work. In addition, aptamer 4 presents one of the lowest percentages in the control tests vs. magnetic beads.

Once the best potential candidates were identified, candidate 4 was synthesized by *Alpha DNA* with the Cy5 modification in 5' and analyzed by microscale thermophoresis; **Figure 4-3** shows the results provided by the device, the K_D value, as well as the saturation results are obtained directly through a report of the device.

The first incubation of the sample (20 minutes) has yielded a K_D similar to that shown in **Figure 4-3**, however there is a variation between the results of each sample, which gives us a signal-to-noise ratio by below the confidence limit for the results (signal-to-noise ratio of 4.0).

After second incubation (24 h) we have obtained a signal-to-noise ratio of 17.9 (**Figure 4-3**).

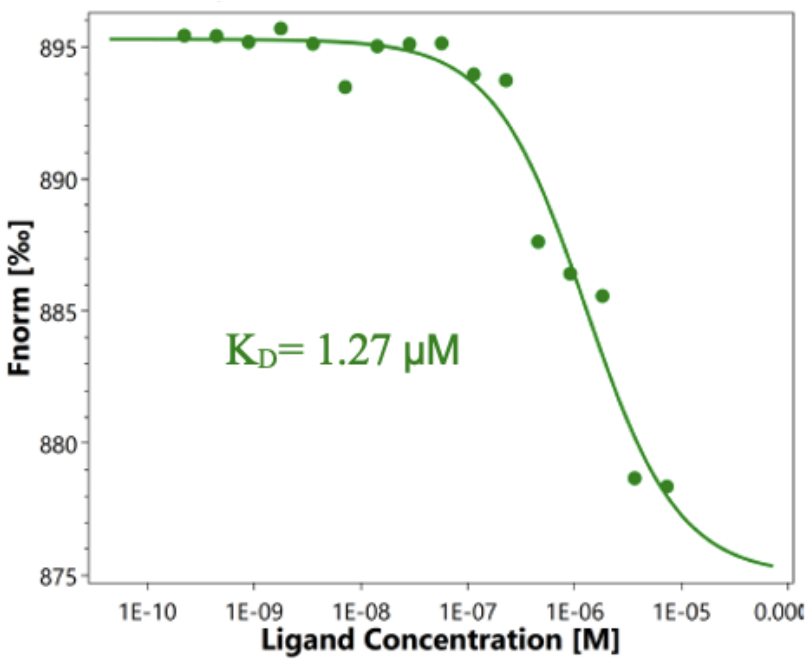


Figure 4-10 Dissociation constant candidate 4

Aptamer candidat 4 labelled with Cy5 at 5', Fnorm: Normalized fluorescence, Ligando: PirAB complex. Interaction strength between aptamer candidate 4 and the PirAB complex by detecting the fluorescence signal emitted by the candidate upon exposure to a temperature change introduced by infrared light laser.

4.2.4 Summary of SELEX results

Table 4-5 shows us a summary of the results obtained through the different approaches of the SELEX methodology.

Table 4-5 Summary of SELEX results

Targets	Total nb cycles	Cycles with PAGE purification	Cycles with nicking digestion	Cycles with lambda digestion	Total reads	Reads with expected size (92 nt)	Sequence nb with <2 reads	Sequences nb with 2 reads	Unique sequences
Vibrio parahaemolyticus (strain EB101)	12	All	All	0	183,974	80%	1940	3944	94116
Vibrio parahaemolyticu (strain EB101)	12	0	All	0	78,809	2%	440	343	10217
Salmonella enterica sero enteritidis	15	Cycle 6 only (when digestion change method)	Cycle 1 to 6	Cycle 7 to 15	40,469	0.8%	157	95	6617
Salmonella enterica sero. enteritidis	15	0	0	All	54,856	0.5%	219	145	5403
Campylobacter jejuni sub. jejuni	15	Cycle 7 to 14	All	0	53,005	0.2%	226	174	3311
Campylobacter jejuni sub. jejuni	15	Cycle 7 to 14	Cycle 7 to 14	Cycle 1 to 6	55,971	0.8%	253	184	5062
Complex PirAB	14	0	0	All	57,570	0.6%	225	157	2893

The different ssDNA production method combinations used were chosen to assess: i- if nicking enzymes required PAGE or not to make ssDNA; ii- if yes, at which cycles; iii- how nicking enzymes compared to lambda nuclease; and iv- also according to which cycles were performed with either.

5 DISCUSSION

5.1 Improvement of the selection method of SELEX

The results of “Novel Strategies to Optimize the Amplification of Single-Stranded DNA” from Frontiers (Nehdi, et al., 2020) show that the ssDNA production with the use of nicking and restriction enzymes is effective thanks to the ease with which it is possible to identify the reverse strand following to the enzymatic cuts that allow easy visualization in gel.

Although, these results show us a correct performance of the digestions the use of nicking digestion as new method for production of ssDNA in SELEX has caused complications during the methodology from the cycle number 7, this shows us a possible interference of the small digested fragments which can quickly “locate” their complementary part and rejoin the ssDNA. In this way, the interaction between the candidates and the ligand is affected by not being able to take the three-dimensional structure that gives the aptamer the possibility to identify the ligand. In principle, gel-based purification could help keep this procedure clean, but it remains a time-consuming step. That was why to produce ssDNA, we have performed, in parallel with gel-based purification, simple denaturation which allowed SELEX iterations to be done much faster. However, this likely caused some selective pressure for shorter sequences which quickly overwhelmed our SELEX and might have even contaminated the SELEX procedure where we did gel purification (a shortage of filter tips for several months probably did not help in that regard).

The use of lambda exonuclease to produce ssDNA during SELEX can speed up the methodology; the SELEX done with this enzymatic digestion type has been carried out in only 6 days, completing a total of 14 SELEX cycles. Of course, total time will depend on the incubation times of the methodology, however even with long incubation times it is possible to perform several SELEX iterations in a few working days.

In summary, the method of ssDNA production by digestion of nicking endonuclease enzymes has been compared against the same ligand in 4 different ways: i- using the gel purification step in all SELEX cycles; where the highest percentage of reads with the expected size (92 nucleotides, **Table 4-5**) was obtained, ii- without purification in any of the SELEX cycles, iii- with purification only in cycle 8, iv- with purification of cycle 8 to cycle 11.

In all the SELEX where the gel purification step was not constant throughout all the cycles, low percentages of sequences of the expected size and a high concentration of non-specific bands/artifacts were obtained.

The rest of the enzyme combinations used in the SELEX for *Salmonella* and *Campylobacter* yielded a similar percentage of sequences of the expected size.

In the case of SELEX against the PirAB toxin, a low percentage of sequences of the expected size was obtained, similar to those of the different combinations of enzymes, however, it was possible to identify a sequence with a certain affinity to the ligand (**Figure 4-3**), although further study is still necessary to improve its characteristics, in addition to the fact that the complete methodology can be carried out in a short period of time.

The improvement of the methodology through a faster method of ssDNA production represents one of the secondary objectives of this work, although it is not involved in the advantages sought for the producers directly on the field of the mentioned industries, it was studied with the purpose to explore new routes for realizing faster SELEX that can be used in different methods for projects involved in aptamer-based identification.

5.2 SELEX against pathogenic bacteria

Candidates have been selected for the identification of *Vibrio parahaemolyticus* by a combination of different types of enzymatic digestion, during some of them there were problems that possibly affected the good development and enrichment of the candidates, the preliminary characterization of these shows an affinity percentage that is not clear enough to define the sequences as true aptamers. In addition, during the course of the following SELEX with different ligands one of the sequences characterized was found as a contaminant in the samples of libraries from all other selections (*Salmonella, Campylobacter* an PirAB SELEX), which lets us think these sequences are unlikely to be aptamers and indicates that we had contamination problems, due to the fact that the DNA template used in the different SELEX was the same and by using tips without filters, the possibilities of contamination between different SELEX samples increased considerably.

The sequences selected for *Campylobacter* and *Salmonella* all have multiple reads (two or more copies) in the sequencing performed, so we chose them as candidate aptamers for further assays. These candidate sequences have been identified with methodologies that use different combinations of enzymatic digestions (**Table 4-2** and **Table 4-3**). With this, we can hope we identified true aptamers with the use of the different digestions, even with the problematic setbacks that we have encountered during experimentation, allow us to enrich the sequences. However, it is likely that some more effective candidates have been lost during the development of the cycles due to the interferences that were already mentioned in **section 5.1** of this chapter.

The sequences selected and characterized for the PirAB complex show an interesting identification response, as well as a possible joint work that gives us the opportunity to expand the type of sensors or methodologies that could be performed for the identification of the ligand in the field, even if further characterization needs to be performed.

All the candidate sequences for characterization were selected based on the number of copies present in the sequencing, this shows us the enrichment of them during the SELEX cycles.

The sequences obtained as candidates can lead the work towards an eventual development of biosensors capable of identifying the ligands of interest in a short analysis time (minutes). For this, it is still necessary to characterize the chosen sequences and, in the case of the PirAB

complex, an optimization of the SELEX conditions to improve the affinity of the potential aptamer found.

The use of this type of biosensors gives us the advantage of rapid identification on the field, which gives producers a greater possibility of carrying out an efficient action plan in the presence of the pathogen that can cause the disease. Although, as mentioned at the beginning of this work, aptamers are not capable of providing us with specific information about the ligand, such as physical or biochemical characteristics, but they do allow us to carry out an efficient and rapid identification of the ligand thanks to the enrichment that is provided by their selection with the SELEX methodology.

6 CONCLUSION AND PERSPECTIVE

6.1 Improvement of the selection method of SELEX

6.1.1 Exonuclease digestion

Lambda exonuclease allowed a much faster progress of SELEX, allowing us to carry out the cycles for more than one ligand at the same time. In addition, preliminary results show us that the use of this type of enzymatic digestion does not present significant problems that could affect the characteristics of the selected candidates, at least not compared to the other methods that we have tried.

However, it is still necessary to verify more specifically common problems such as the presence of primer dimers that appear during the final cycles of the methodology, this fact may or may not be related to the type of digestion used. Even so, the selected candidates are apparently unaffected by their presence. The primer dimers were most detrimental to sequencing, since the presence of primer dimers in large quantities limited the number of readings of good sequences, thus limiting our ability to use deep sequencing results to identify the best candidates and may have caused additional biases/artefacts for the bioinformatic analysis.

6.1.2 Endonuclease digestion

The use of a combination of endonucleases as a method to cleave the reverse strand has caused complications during the methodology in the last SELEX cycles, when the abundance of copies of each candidate sequences is more important and when the remaining small fragments of digested DNA have more chances of finding their complementary part and join again. It is therefore recommended to use this digestive method as a tool in the production of ssDNA only in the first cycles (cycle 0 to cycle 6) when the number of copies of the same sequence is not yet found in the sample in high abundance.

Even if the presented method has shown the appearance of artefacts during the development of the methodology and has caused some drawbacks, the fact that it does not require chemical modifications could make it a convenient strategy for some approaches. For instance, the fact that this approach allows the presence of a 5' phosphate (on its forward/aptamer strand) represents an advantage for certain applications, for example, the possibility of circularizing the library after PCR amplification.

In general, if we compare the ssDNA production methods based on their ease of execution, digestion with lambda exonuclease is carried out simply and quickly compared to digestion with nicking endonucleases; cutting with endonucleases also needs more consumables (four enzymes), however it allows the verification of the correct cut because we can add fluorescent labeling in the complementary strand, contrary to lambda exonuclease where we must modify the complementary strand with the phosphate group so that the enzyme can recognize it to degrade it.

That said, it will depend on the approach of the method used to select the ssDNA production method that best suits the project needs.

6.2 SELEX against pathogenic bacteria

6.2.1 Whole bacteria as a target

In principle, the use of whole bacteria as a target should allow to select aptamers able to identify the pathogen quickly and, within an eventual biosensor, allow us to conclude whether or not there is a risk of contamination. The limitation in terms of the selection with this type of targets is its manipulation, this limits the possibilities of characterization of the candidates (e.g. it is not possible to have molar concentrations of bacteria). The candidates selected in this work require further characterization to be able to define them or not as aptamers; as can be seen in **Figure 4-3** for example, the results of SELEX against *Vibrio parahaemolyticus* are not entirely clear and it is still necessary to characterize the candidates for a verification of affinity or a methodology improvement.

6.2.2 Toxin complex as a target

The selection of aptamers for the PirAB complex as a target gives us the possibility of having a more specific identification for the animal health problem caused by *Vibrio* species carrying the PirAB toxin, like *Vibrio parahaemolyticus*; because it is a specific target of the pathogenic strain causing the disease attacking shrimp farming. Identification becomes more reliable and gives us the possibility of having enough time in the field to perform corrective actions that reduce the losses caused by this pathogen or can be used in surveillance programs.

The sequences selected and characterized *in vitro* show a possible positive behavior as aptamers, however it is still necessary to carry out studies in controlled conditions to more fully characterize chosen aptamers (e.g., specificity tests) as well as optimize the sandwich assay.
The K_D obtained shows us the potential of the sequence candidate 4 as an aptamer, however, the dissociation that it represents is not the desired one, in view of which it is required to make improvements in the experiments by making the conditions stricter (e.g., increasing the wash time, change negative selections) to improve this K_D and have an aptamer with a better affinity.
Some experiments were added with the aim of improving the characteristics of the candidates against the complex (cycles 15 to 17 **Table 8-2**), the samples will be sent to a new sequencing and a new bioinformatics analysis, as well as a new candidate's characterization will still be needed.

Eventually, assays should be performed directly on samples of the animal to define whether the sequences are viable aptamers or not that meet the objective of identification necessary to improve conditions in the field.

The use of microscale thermophoresis as a potential alternative for the determination of K_Ds in aptamers represents a simple tool to use and low consumption of materials, since the sample needs are of very low volume and the results are obtained in a matter of minutes. However, there are multiple conditions to optimize during reads; such as the incubation time for a good response of the device, the detergents and their concentrations to be used in the samples to avoid aggregations, changes or improvements in the buffer for a correct reading. All these optimizations come to extend the time of experimentation and the needs of consumables; however, it can be a useful alternative depending on the focus of the project.

7 References

Agency, C. F. (2020). *Canadian Food Inspection Agency*. Récupéré sur https://www.inspection.gc.ca/about-cfia/transparency/regulatory-transparencyand-openness/food-safety-investigations/eng/1332299626115/1332299812611

Aguilar-Rendón, K. G., Lozano-Olvera, R., Yáñez-Rivera, B., & Soto-Rodriguez, S. A. (2020). Bacteriological and histopathological analysis of Penaeus vannamei experimentally infected with Vibrio parahaemolyticus-AHPND strains. *Diseases of Aquatic Organisms, 140*, 167-177.

Bonami, J., & Ronan, Q. (2016). Simultaneous detection of multiple pathogens (WSSV y IHHNV). *AquaTIC*.

Boonthai, T., Vuthiphandchai, V., & Nimrat, S. (2011). Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (Penaeus monodon). *Aquaculture Nutrition, 17(6)*, 634-644.

Cervantes-García, E. (2020). Campylobacter: emerging or reemerging. . *Rev Mex Patol Clin Med Lab. 67 (3)*, 142-149.

Chen, M., Yu, Y., Jiang, F., Zhou, J., Li, Y., Liang, C., . . . Zhang, G. (2016). Development of Cell-SELEX Technology and Its Application in Cancer Diagnosis and Therapy. *International journal of molecular sciences, 17(12)*, 2079.

Chong, C., & Low, C. (2019). Synthetic antibody: Prospects in aquaculture biosecurity. *Fish & shellfish immunology, (86)*, 361-367.

D. M. Morens, G. K., & Fauci, A. S. (2004). The challenge of emerging and re-emerging infectious diseases. *Nature, 430 (6996)*, 242-249.

Darmostuk, M., Rimpelova, S., Gbelcova, H., & Ruml, T. (2015). Current approaches in SELEX: An update to aptamer selection technology. *Biotechnology advances, 33(6)*, 1141-1161.

Delgado, C. L. (2003). Fish to 2020: Supply and demand in changing global markets. *WorldFish*, Vol. 62.

Dhar, A. K., Cruz-Flores, R., Caro, L. F., Siewiora, H. M., & Jory, D. (2019). Diversity of single-stranded DNA containing viruses in shrimp. *VirusDisease*, 43-57.

Duan, N., Wu, S., Chen, X., Huang, Y., Xia, Y., Ma, X., & Wang, Z. (2013). Selection and characterization of aptamers against Salmonella typhimurium using whole-bacterium systemic evolution of ligands by exponential enrichment (SELEX). . *Journal of agricultural and food chemistry, 61 (13)*, 3229-3234.

Ellington, A. D., & Szostak, J. W. (1990). n vitro selection of RNA molecules that bind specific ligands. *Nature 346 (6287)*, 818-822.

FAO. (2018). The state of world fisheries and aquaculture .

Flegel, T. W. (2019). A future vision for disease control in shrimp aquaculture. *Journal of the World Aquaculture Society*, 249-266.

Flegel, T. W., & Lo, C. F. (2014). Free release of primers for specific detection of bacterial isolates that cause acute hepatopancreatic necrosis disease (AHPND). *Network of Aquaculture Centres in Asia and the Pacific, Bangkok, Thailand.*

Fonkwo, P. N. (2008). Pricing infectious disease: The economic and health implications of infectious diseases. *EMBO reports, 9(S1)*, S13-S17.

González, C. A. (2010). Aquatic animal pathogens of public health and aquaculture interest.

Halwart, M. S. (2009). Aquaculture en cage. Etudes regionales et aperçu mondial. *FAO*.

Han, J. E., Tang, K. F., Lightner, D. V., & Tran, L. (2015). Photorhabdus insect related (Pir) toxin-like genes in a plasmid of Vibrio parahaemolyticus, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis. Aquat. Org., 113*, 33-40.

Hernández, F. J., & Hincapié, J. A. (2012). Aptamers: diagnostic and therapeutic agents. *Latreia, 25(2)*, 159-168. .

Hernández-Rodríguez, A., Alceste-Oliviero, C., Sanchez, R., Jory, D., Vidal, L., & Constain-Franco, L. F. (2001). Aquaculture Development Trends in Latin America and the Caribbean. *Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium*, 317-340.

Hoinka, J., Backofen, R., & Przytycka, T. (2018). AptaSUITE: a full-featured bioinformatics framework for the comprehensive analysis of aptamers from HT-SELEX experiments. *Molecular Therapy-Nucleic Acids 11*, 515-517.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B. E., & Kautsky, N. (2003). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science & Technology*, 255-266.

Jajere, S. M. (2019). A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance . *Veterinary world, 12(4)*, 504.

Joshi, J., Srisala, J., Truong, V. H., Chen, I. T., Nuangsaeng, B., Suthienkul, O., . . . Thitamadee, S. (2014). Variation in Vibrio parahaemolyticus isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, 297-302.

Knodler, L. A., & Elfenbein, J. R. (2019). Salmonella enterica. *Trends in microbiology, 27(11)*, 964-965.

Lara, M., Torres, M., Baez, M., & Albertini, S. (2019). GENERAL ASPECTS OF ANTIMICROBIALS USE AND THEIR INTERACTION WHIT THE ENVIRONMENT: AN EMERGING PROBLEM. *COMPENDIUM OF VETERINARY SCIENCES*, 24-37.

Lee, C. T., Chen, I. T., Yang, Y. T., Ko, T. P., Huang, Y. T., Huang, J. Y., . . . Lo, C. F. (2015). The opportunistic marine pathogen Vibrio parahaemolyticus becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proceedings of the National Academy of Sciences, 112(34)*, 10798-10803.

Li, P., Yan, Y., Wei, S., Wei, J., Gao, R., Huang, X., . . . Qin, Q. (2014). Isolation and characterization of a new class of DNA aptamers specific binding to Singapore grouper iridovirus (SGIV) with antiviral activities. . *Virus research, 188*, 146-154.

Li, P., Zhou, L., Wei, J., Yu, Y., Yang, M., Wei, S., & Qin, Q. (2016). Development and characterization of aptamer-based enzyme-linked apta-sorbent assay for the detection of Singapore grouper iridovirus infection. *Journal of applied microbiology, 121(3)*, 634-643.

Lightner, D., & Pantoja, C. (2003). Manual for the diagnosis of shrimp diseases. *Programa de reconstrucción Huracán Match, United States Department of Agriculture.* .

Love, D. C., Fry, J. P., Cabello, F., Good, C. M., & Lunestad, B. R. (2020). Veterinary drug use in United States net pen Salmon aquaculture: Implications for drug use policy. *Aquaculture*, 734820.

Marimuthu, C., Tang, T. H., Tominaga, J., Tanc, S. C., & Gopinath, S. C. (2012). Single-stranded DNA (ssDNA) production in DNA aptamer generation. *Analyst, 137(6)*, 1307-1315.

Nehdi, A., Samman, N., Aguilar-Sánchez, V., Farah, A., Yurdusev, E., Boudjelal, M., & Perreault, J. (2020). Novel Strategies to Optimize the Amplification of Single-Stranded DNA. *Frontiers in Bioengineering and Biotechnology*, 401.

Oddsson, G. V. (2020). A Definition of Aquaculture Intensity Based on Production Functions—The Aquaculture Production Intensity Scale (APIS). *Water, 12 (3)*, 765.

OIE, W. O. (2020). Manual of diagnostic tests for aquatic animals.

Peña-Navarro, N., Vargas-Cordero, R., & Varelas-Mejía, A. (2013). Natural products as stimulators of the immune system of Litopenaeus vannamei, infected with Vibrio parahaemolyticus. *Agronomía Mesoamericana* , 133-147.

Ponce, M. B. (2015). Aptamers for the detection of small organic molecules. (Doctor of Science and Technology). . *Universidad Nacional de General Sarmiento, (1178/11)*.

Punnarak, P., Santos, M. D., Hwang, S. D., Kondo, H., Hirono, I., Kikuchi, Y., & Aoki, T. (2012). RNA aptamers inhibit the growth of the fish pathogen viral hemorrhagic septicemia virus (VHSV). *Marine biotechnology, 14(6)*, 752-761.

Rodríguez-Camacho, J., Méndez-Goméz, E., Rivas-Montaño, A., & Cortés-Ruiz, J. (2014). Evaluation of the presence of Vibrio parahaemolyticus in white shrimp (Litopenaeus vannamei) estuarine-wild from southern Sinaloa and northern Nayarit by microbiological analysis and PCR. *Revista Bio Ciencias*, 281-292.

Rojas A., W. S. (2009). Étude sur l'aquaculture en cage: l'Amérique latine et les Caraïbes. *Aquaculture en cage: Études régionales et aperçu mondial. FAO Document technique sur les pêches*, 73-104.

Salas, R. G., Río, M. V., & Concepción, I. P. (2021). INTENSIVE USE OF PROPHYLACTIC ANTIBIOTICS IN AQUACULTURE: A GROWING PROBLEM FOR HUMAN AND ANIMAL HEALTH. *Revista Universidad y Sociedad*, 204-210.

Shamah, S. M., Healy, J. M., & Cload, S. T. (2008). Complex target SELEX. *Accounts of chemical research, 41(1)*, 130-138.

Shinn, A. P., Pratoomyot, J., Griffiths, D., Trong, T. Q., Vu, N. T., Jiravanichpaisal, P., & Briggs, M. (2018). Asian Shrimp production and the economic costs of disease. *Asian Fisheries Science*, 29-58.

Sirikharin, r., S. Taengchaiyaphum, P. S., Chi, T. d., Mavichak, R., Proespraiwong, p., Nuangsaeng, B., . . . Sritunyalucksana, K. (2015). Characterization and PCR Detection Of Binary, Pir-Like Toxins from Vibrio parahaemolyticus Isolates that Cause Acute Hepatopancreatic Necrosis Disease (AHPND) in Shrimp. *PloS one, 10(5)*, e0126987.

Sirikharin, R., Taengchaiyaphum, S., Sritunyalucksana, K., Thitamadee, S., Flegel, T., Mavichak, R., & Proespraiwong, P. (2014). A new and improved PCR method for detection of AHPND bacteria. *Network of Aquaculture Centres in Asia and the Pacific*.

Snelling, W., Matsuda, M., Moore, J., & Dooley, J. (2005). Campylobacter jejuni. *Applied microbiology, 41*, 297-302.

Soto-Rodriguez, S. A., Gómez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., & Morales-Covarrubias, M. S. (2015). Field and Experimental Evidence of Vibrio parahaemolyticus as the Causative Agent of Acute Hepatopancreatic Necrosis Disease of Cultured Shrimp (Litopenaeus vannamei) in Northwestern Mexico. *Applied and environmental microbiology*, 1689-1699.

Soto-Rodriguez, S. A., Gómez-Gil, B., Lozano-Olvera, R., Bolan-Mejia, C., & Encino-Ibarra, K. G.-R. (2018). Pathological, Genomic and Phenotypical Characterization of Vibrio

parahaemolyticus, Causative Agent of Acute Hepatopancreatic Necrosis Disease (AHPND) in Mexico. *Asian Fisheries Science*, 102-111.

Sua, Y. C., & Liub, . (2007). Vibrio parahaemolyticus: A concern of seafood safety . *Food microbiology, 24(6)*, 549-558.

Svobodová, M., Pinto, A., Nadal, P., & Sullivan, C. K. (2012). Comparison of different methods for generation of single-stranded DNA for SELEX processes. *Analytical and bioanalytical chemistry, 404(3)*, 835-842.

Tiwongger, S., Proespraiwong, P., Thawonsuwan, J., Sriwanayos, P., Kongkumnerd, J., Chaweepack, T., . . . Hirono, I. (2014). Development of PCR diagnosis method for shrimp acute hepatopancreatic necrosis disease (AHPND) strain of Vibrio parahaemolyticus. *Fish Pathol., 49*, 159-164.

Tuerk, C., & Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *science, 249(4968)*, 505-510.

Turco, M., Rodrigo, V., Sabbaj, L., & MS, V. R. (2014). Campylobacter gastroenteritis jejuni and Campylobacter coli in a pediatric hospital. *Hosp. Niños*, 149-155.

Varela-Mejías, A., & Peña-Navarro, N. (2014). arly Mortality Syndrome (EMS / AHPNS) in farmed shrimp: A review. *Repertorio Científico, 17(1)*, 25-30.

Varelas-Mejía, A., Peña-Navarro, N., & Aranguren-Caro, L. F. (2017). Acute necrosis of the hepatopancreas: a review of the disease in Penaeus vannamei. *Agronomía Mesoamericana, 28(3)*, 735-745.

WHO. (1999). Removing Obstacles to Healthy Development. *Geneva, Switzerland: World Health Organization*.

WHO. (2018, February 20). *World Health Organization*. Récupéré sur World Health Organization: https://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal)

WHO. (2020, May 1). Récupéré sur World Health Organization: https://www.who.int/es/news-room/fact-sheets/detail/campylobacter

Wua, Y. X., & Kwon, Y. J. (2016). Aptamers: The ‘‘evolution” of SELEX. *Methods, 106*, 21-28.

Wurmann, C. (2019). Aquaculture in Latin America and the Caribbean: Progresses, opportunities and challenges. *Aquatechnica: Revista Iberoamericana de Acuicultura*, 1-21.

Wurmann, C. G., Madrid, R. M., & Burgger, A. M. (2004). Shrimp farming in Latin America: Current status, opportunities, challenges and strategies for sustainable development. *Aquaculture Economics & Management*, 117-141.

Xiao, J., Liu, L., Ke, Y., Li, X., Liu, Y., Pan, Y., . . . Wang, Y. (2017). Shrimp AHPND-causing plasmids encoding the PirAB toxins as mediated by pirAB-Tn903 are prevalent in various Vibrio species . *Scientific reports, 7 (1)*, 1-11.

Yu, Q., Liu, M., Wei, S., Qin, X., Qin, Q., & Li, P. (2021). Research progress and prospects for the use of aptamers in aquaculture biosecurity. *ELSEVIER Aquaculture*.

Yu, Q., Liu, M., Wei, S., Wu, S., Xiao, H., Qin, X., . . . Li, P. (2019). Characterization of ssDNA aptamers specifically directed against Trachinotus ovatus NNV (GTONNV)-infected cells with antiviral activities. *Journal of General Virology, 100(3)*, 380-391.

Yu, Y., Huang, Y., Ni, S., Zhou, L., Liu, J., Zhang, J., . . . Qin, Q. (2017). Singapore grouper iridovirus (SGIV) TNFR homolog VP51 functions as a virulence factor via modulating host inflammation response. *Virology, 511*, 280-289.

8 APPENDIX I

Table 8-1 SELEX conditions, whole bacteria as a target

Cycle	Incubation time (minutes)	Positive selection (target volume, µl)	Negative selection (target volume (µl)/type)	Digestion type	Gel purification (PAGE)
Vibrio parahaemolyticus (PAGE)					
1-3	60	200	---	Nickase	+
4	60	200	200/magnetic beads	Nickase	+
5	45	100	100/S*almonella enteritidis*	Nickase	+
6	45	100	100/*Vibrio cholerae*	Nickase	+
7	20	50	50/*Vibrio alginolyticus*	Nickase	+
8	20	50	50/*Vibrio vulnificus*	Nickase	+
9	20	50	50/ *Vibrio alginolyticus*	Nickase	+
10	15	50	50/ magnetic beads	Nickase	+
11	15	50	50/ *Vibrio cholerae*	Nickase	+
12	15	50	50/ *Vibrio alginolyticus*	---	---
Vibrio parahaemolyticus (Heating)					
1-3	60	200	---	Nickase	-
4	60	200	200/magnetic beads	Nickase	-
5	45	100	100/S*almonella enteritidis*	Nickase	-
6	45	100	100/*Vibrio cholerae*	Nickase	-
7	20	50	50/*Vibrio alginolyticus*	Nickase	-
8	20	50	50/*Vibrio vulnificus*	Nickase	-
9	20	50	50/ *Vibrio alginolyticus*	Nickase	-
10	15	50	50/ magnetic beads	Nickase	-
11	15	50	50/ *Vibrio cholerae*	Nickase	-
12	15	50	50/ *Vibrio alginolyticus*	---	---
Salmonella enterica sero enteritidis (Heating/Lambda)					
1	60	200	---	Lambda	-
2	60	100	---	Lambda	-
3	60	100	---	Lambda	-
4	45	100	100/magnetic beads	Lambda	-
5	45	20	20/S*almonella choleraesuis subsp. choleraesuis*	Lambda	-

6	30	20	20/ S*almonella enterica subsp. enterica serovar Paratyphi*	Lambda	-
7	30	20	20/ S*almonella enterica subsp. enterica serovar Dublin*	Lambda	-
8	20	20	20/ S*almonella paratyphi*	Lambda	-
9	20	20	20/ All mentioned	Lambda	-
10	20	20	20/ All mentioned	Lambda	-
11	20	10	10/ All mentioned	Lambda	-
12	15	10	10/ All mentioned	Lambda	-
13	15	10	10/ All mentioned	Lambda	-
14	15	10	10/ All mentioned	Lambda	-
15	10	10	10/ All mentioned	---	---
Salmonella enterica sero enteritidis (Heating/Nickase+Lambda)					
1	60	200	---	Nickase	-
2	60	100	---	Nickase	-
3	60	100	---	Nickase	-
4	45	100	100/magnetic beads	Nickase	-
5	45	20	20/S*almonella choleraesuis subsp. choleraesuis*	Nickase	-
6	30	20	20/ S*almonella enterica subsp. enterica serovar Paratyphi*	Nickase	+
7	30	20	20/ S*almonella enterica subsp. enterica serovar Dublin*	Lambda	-
8	20	20	20/ S*almonella paratyphi*	Lambda	-
9	20	20	20/ All mentioned	Lambda	-
10	20	20	20/ All mentioned	Lambda	-
11	20	10	10/ All mentioned	Lambda	-
12	15	10	10/ All mentioned	Lambda	-
13	15	10	10/ All mentioned	Lambda	-
14	15	10	10/ All mentioned	Lambda	-
15	10	10	10/ All mentioned	---	---
Campylobacter jejuni sub. jejuni (PAGE/Heating/Nickase)					
1	60	200	---	Nickase	-
2	60	100	---	Nickase	-

3	60	100	---	Nickase	-
4	45	20	100/ magnetic beads	Nickase	-
5	45	20	20/ Campylobacter jejuni subsp. doylei	Nickase	-
6	30	20	20/ Campylobacter coli	Nickase	-
7	30	20	20/ Campylobacter jejuni subsp. doylei	Nickase	+
8	20	20	20/ All mentioned	Nickase	+
9	20	20	20/ All mentioned	Nickase	+
10	20	20	20/ magnetic beads	Nickase	+
11	20	10	10/ All mentioned	Nickase	+
12	15	10	10/ All mentioned	Nickase	+
13	15	10	10/ All mentioned	Nickase	+
14	15	10	10/ All mentioned	Nickase	+
15	10	10	10/ All mentioned	---	---
Campylobacter jejuni sub. jejuni (PAGE/Heating/Lambda/Nickase)					
1	60	200	---	Lambda	-
2	60	100	---	Lambda	-
3	60	100	---	Lambda	-
4	45	20	100/ magnetic beads	Lambda	-
5	45	20	20/ Campylobacter jejuni subsp. doylei	Lambda	-
6	30	20	20/ Campylobacter coli	Lambda	+
7	30	20	20/ Campylobacter jejuni subsp. doylei	Nickase	+
8	20	20	20/ All mentioned	Nickase	+
9	20	20	20/ All mentioned	Nickase	+
10	20	20	20/ magnetic beads	Nickase	+
11	20	10	10/ All mentioned	Nickase	+
12	15	10	10/ All mentioned	Nickase	+
13	15	10	10/ All mentioned	Nickase	+
14	15	10	10/ All mentioned	Nickase	+
15	10	10	10/ All mentioned	---	---

Table 8-2 SELEX conditions, PirAB complex as a target

Cycle	Heating elution after Positive selection (94 C/5 min)	Incubation time (minutes)	Positive selection (target volume, µl)	Negative selection	Digestion type
PirAB complex (Heating/Lambda)					
1	-	60	200	---	Lambda
2	-	60	100	---	Lambda
3	-	60	50	---	Lambda
4	-	45	20	20 µl/magnetic beads	Lambda
5	+	45	20	Lysozyme/BSA 0.25 µM	Lambda
6	+	30	20	Lysozyme/BSA 0.25 µM	Lambda
7	+	30	20	Lysozyme/BSA 0.25 µM	Lambda
8	+	30	20	Lysozyme/BSA 0.25 µM	Lambda
9	+	20	20	Lysozyme/BSA 0.25 µM	Lambda
10	+	20	15	Lysozyme/BSA 0.5 µM	Lambda
11	+	20	15	Lysozyme/BSA 0.5 µM	Lambda
12	+	15	10	Lysozyme/BSA 0.5 µM	Lambda
13	+	15	10	Lysozyme/BSA 0.5 µM	Lambda
14	+	10	5	Lysozyme/BSA 0.5 µM	---
Recombinant *PirA* (Heating/Lambda)					
15	+	10 + 60 washing (x2)	5	Lysozyme/BSA 0.5 µM	Lambda
16	+	10+ 60 washing (x2)	5	Lysozyme/BSA 0.5 µM	Lambda
Recombinant *PirB* (Heating/Lambda)					
15	+	10 + 60 washing (x2)	5	Lysozyme/BSA 0.5 µM	Lambda
16	+	10+ 60 washing (x2)	5	Lysozyme/BSA 0.5 µM	Lambda
PirAB complex (Heating/Lambda)					
17*	+	10+ 60 washing (x2)	5	Lysozyme/BSA 0.5 µM	---

*Cycle 17: Cycle 16 of recombinant PirA and PirB were mixed to continue with the final cycle against the PirAB complex.